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DEVELOPMENT OF OIL IN THE SEED OF ASCLEPIAS SYRIACA L.[1]

C. Y. HOPKINS AND MARY J. CHISHOLM

Abstract

Seed of *Asclepias syriaca* L. was gathered at intervals during the growing season. Analyses of the seed, the oil, and the fatty acids were made, the latter by gas chromatography. About 75% of the total oil was formed in the first 5 weeks after flowering and the remaining 25% over the final 7 weeks. There was a sudden change in fatty acid composition at a middle stage of development, resulting in a fall in the percentage of hexadecenoic and octadecenoic acids and a rise in linoleic acid. Thereafter the changes were small except that linoleic showed a further increase in proportion to the total fatty acids.

The weight of individual fatty acids per 1000 seeds was calculated at each stage of development. Corresponding to the change in percentage composition, there was a sharp increase in the weight of linoleic acid per 1000 seeds and an actual decrease in the weight of hexadecenoic and octadecenoic acids, between the 5th and 7th week after flowering. Between the 7th week and maturity, linoleic acid increased considerably, hexadecenoic and octadecenoic acids increased slightly, and the others remained unchanged in amount. There was a net loss of hexadecenoic acid from the 5th week to maturity. It is postulated that 9-hexadecenoic acid was converted in the seed to 11-octadecenoic acid and that oleic acid was converted to linoleic acid.

Introduction

Relatively little is known of the mechanism of synthesis of glycerides and their component fatty acids in plants. One approach to the problem has been to examine the characteristics and composition of a seed oil at various stages of maturity of the seed. This procedure shows promise of providing useful information if the analysis is specific for the individual fatty acids and adequate to determine the weight of each acid in a stated number of seeds. It is desirable that this method be applied to a number of widely differing species of seeds which produce different types of oil.

A native species of perennial plant, *Asclepias syriaca* L., was chosen for such a study. The seed is of moderate size, easy to collect, and contains a fair

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proportion of oil. It develops and ripens uniformly. A detailed analysis of the oil, already reported (1), showed it to be of unusual composition, containing saturated, monoene, and diene acids of both the C_{16} and C_{18} series. Gas–liquid chromatography was employed in the present work for the quantitative determination of the individual fatty acids throughout the period of their formation in the seed. Final calculations were made on the basis of the weight of each component per 1000 seeds.

Earlier Work

Early investigations of the development of oil in plant species were limited to determination of the percentage of oil in the seed and the constants of the oil. A number of these studies have been reviewed by Hilditch (2). The more recent work has given useful data on the rate of formation of some individual acids (combined as glycerides). In the cottonseed, Grindley (3) found that very little fatty acid was formed up to the 30th day after flowering but from then until the 60th day the saturated, oleic, and linoleic acids increased steadily in amount when calculated as grams of acid per 100 seeds. A similar increase in actual amounts of oleic, linoleic, and linolenic acids was seen in the soybean by Simmons and Quackenbush (4). In the palm kernel (5), Crombie observed the same pattern of a general increase in amount of each fatty acid but a more rapid accumulation of saturated acids, especially lauric, as compared to oleic and linoleic acids. Determination of the fatty acid composition was carried out in the above studies by the thiocyanate method, by ultraviolet absorption analysis, or by column chromatography.

Methods and Results

Collection and Analysis of Seed

Seed was obtained from a stand of plants near Wrightville, Que., in 1959. Flowering was general on July 12 and pods were forming by July 18. The first collection of pods was on August 18 and the last on October 6, at which time the pods appeared to be fully ripe and were opening to allow the seed to disperse. Fifty pods were collected each time. Not more than three were taken from one plant and selection was at random except that pods of approximately the same size and apparent maturity were taken. The seeds were not allowed to ripen after picking. They were removed from the pods on the same day, weighed, dried at 110°, and ground in a Wiley mill.

The oil was extracted quantitatively in a Soxhlet apparatus with petroleum ether (b.p. 30–60°). Moisture and oil content of the seed from the 1959 crop are shown in Table I. The moisture content fell steadily during maturation to approximately 40%. Although this seems high for a ripe seed it was not considered feasible to allow the pods to ripen further on the plants because of the danger of losing the seed by dispersal.

The oil content, calculated as percentage of total dry matter (column 6), varied somewhat from week to week but did not increase in percentage. This result suggests that the oil was synthesized at about the same rate as the other

TABLE I

Composition of seed, 1959 crop

Date of harvest	Age of seed, weeks*	Wt. of 1000 seeds, as received, g	Moisture, wt.%	Oil		
				As received, wt.%	Dry basis, wt.%	Per 1000 seeds, g
Aug. 18	5	12.1	66.6	7.2	21.6	0.87
Sept. 1	7	10.3	53.3	8.5	18.1	0.88
Sept. 9	8	10.6	48.9	†	†	†
Sept. 15	9	8.4	45.0	11.5	20.9	0.97
Sept. 22	10	8.4	40.1	13.1	21.9	1.10
Sept. 29	11	9.5	39.6	11.7	19.4	1.12
Oct. 6	12	9.0	40.8	12.8	21.6	1.15

*From date of flowering.
†A portion of this sample was lost.

components of the seed. Thus it does not seem that any intermediate substance was laid down in the seed during the early part of the period under study and later converted to oil. When calculated as weight of oil per 1000 seeds (column 7), a steady but rather limited increase in actual oil content is apparent. About 75% of the total oil was formed prior to the harvesting of the first lot of seed, viz., in the 5 weeks after flowering, and the remaining 25% was formed over the succeeding period of 7 weeks.

Analysis of Oil

The analytical values for the oil were determined by official A.O.C.S. methods (6) and are given in Table II. The free titratable acid was low throughout and decreased to a content of 0.7%, calculated as oleic acid. Iodine value rose from an average of 120 in the 1st month to an average of 127 in the final 3 weeks, evidently due to an increase in diene content. The proportion of unsaponifiable matter was practically unchanged throughout.

TABLE II

Characteristics of the oil, 1959 crop

Age of seed, weeks	Iodine value	Free acid, as oleic, wt.%	Unsap., wt.%	Total diene acids,* wt.%
5	117.0	2.7	2.4	36.8
7	120.5	1.8	2.9	43.4
8	123.0	1.0	2.3	44.9
9	118.4	0.6	2.4	43.6
10	128.4	1.0	2.1	47.4
11	127.2	0.7	2.2	47.4
12	126.8	0.7	2.3	46.1

*As percentage of the oil.

Composition of the Fatty Acids

The oils were saponified and the unsaponifiable matter was removed. The mixed fatty acids were converted to methyl esters and analyzed by gas chromatography. The liquid phase was a succinic acid – diethyleneglycol polyester.

Column length was 1 meter, temperature 190°, and helium flow rate 95 ml/minute, at an inlet pressure of 12.5 p.s.i. The detector was a thermistor type of thermal conductivity cell and the recorder span was 0–5 millivolts. Four chromatograms were made from each lot of esters and the peak areas were measured. The means of these results are shown in Table III.

The fatty acids of the oils consisted almost entirely of those listed in Table III, as reported previously (1). The octadecenoic acids consisted of equal parts of oleic and 11-octadecenoic acids. No isomers of linoleic acid were found (1). The chromatograms showed minor proportions of stearic acid (less than 1%) and linolenic acid (less than 0.5%). Confirmation of their occurrence was obtained by chromatographing the last sample with a 2.5-meter column at 204°.

TABLE III

Percentage composition of the fatty acids, 1959 crop (by gas chromatography*)

Age of seed, weeks	Palmitic, %	Hexadecenoic, %	Hexadecadienoic, %	Octadecenoic, %	Linoleic, %
5	6.3	18.3	3.0	34.6	36.8†
7	5.1	12.1	2.3	31.9	47.6
8	4.6	13.4	2.1	31.3	47.6
9	4.8	12.6	2.2	31.5	47.9
10	4.7	10.7	2.2	29.1	52.3
11	4.2	10.9	1.9	30.4	51.6
12	4.1	11.8	1.9	29.3	52.0

*Probable limit of error, ±0.5 unit per cent.
†The remaining 1% is accounted for by stearic and linolenic acids.

Ultraviolet absorption analysis showed that the content of conjugated acids was negligible (less than 0.2%). It was determined by gas chromatography that there were no acids of chain length C_{10}–C_{15} and none of chain length greater than C_{18} up to C_{22}. A test for oxirane oxygen was negative, showing that epoxy acids were absent. A gas chromatogram of the fully hydrogenated acids did not reveal the presence of any acids other than straight chain C_{16} and C_{18}.

It is evident from Table III that a notable change in percentage composition occurred between the first and second collections, that is between the 5th and 7th week after flowering. The percentage of linoleic acid in the total acids increased markedly while the percentages of the others decreased, particularly hexadecenoic acid. From the 7th week to maturity there was a similar trend although the extent of the change was much less.

Amounts of Individual Acids per 1000 Seeds

The rate of accumulation of the individual acids was calculated from the data in Tables I and III and is shown in Fig. 1. The content of total fatty acids was calculated from the content of oil per 1000 seeds (Table I) and the mean molecular weight of the mixed fatty acids. There was a moderate increase in the weight of total fatty acids over the period studied. Nearly all of the increase was accounted for by linoleic acid, which almost doubled in amount. The

octadecenoic acids increased by about 10% but the C_{16} acids showed a net decrease.

From the 7th to the 10th week there was a general increase in weight of the C_{18} acids, particularly of linoleic acid (Fig. 1). After the 10th week the accumulation of oil became much slower but there was a slight increase in weight of hexadecenoic, octadecenoic, and linoleic acids. Evidently the seed was in the final stage of maturity in this period.

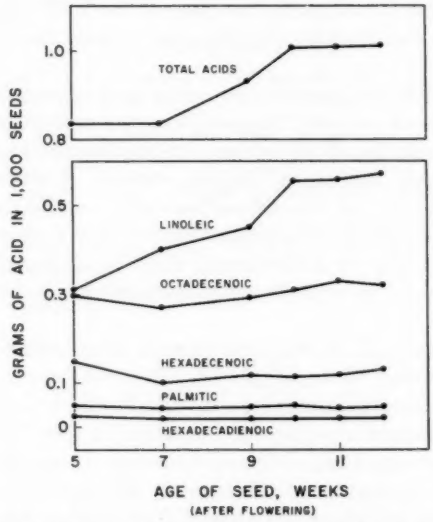

FIG. 1. Weight of individual acids in 1000 seeds at various stages of maturity.

Examination of Seed Collected in 1958

In the 1958 growing season a similar study was carried out with seed collected once a week for 4 weeks. The results were much the same as in 1959. Between the first and second seed collections, the percentage of linoleic acid in the total fatty acids increased from 30.9 to 46.0, while hexadecenoic acid decreased from 19.0 to 13.3 and octadecenoic acid decreased from 42.5 to 32.1. The actual weight of the acids in 40 pods of seeds varied in the same manner; linoleic acid increased from 2.12 to 3.60 g while hexadecenoic and octadecenoic acids decreased from 1.31 to 1.04 g and from 2.92 to 2.51 g respectively.

It was found in a single experiment that the oil in seeds detached from the plant at an early stage underwent a change in composition similar to that in seed allowed to mature on the plant. A quantity of seed from the first 1958 collection, air-dried and stored in the laboratory in paper bags for 3 months, yielded oil which had nearly the same fatty acid composition as the oil from seed collected at full maturity.

Discussion and Conclusions

The most significant change in oil composition during the development of the seed occurred between the 5th and 7th weeks after flowering in the 1959 crop. It consisted of a large increase in the actual amount of linoleic acid and a concurrent decrease in actual amounts of hexadecenoic and octadecenoic acids (all as glycerides) (Fig. 1). The greatest decrease was in 9-hexadecenoic acid and there can be little doubt that there was a disappearance of a considerable proportion of the 9-hexadecenoic acid previously formed. This was confirmed by a similar decrease in amount in the seed of the 1958 crop. The decrease in octadecenoic acids, also observed in both crops, was smaller in proportion to the original amount.

It is unlikely that the 9-hexadecenoic acid was converted to linoleic since this would require addition of two CH_2 groups at the methyl end of the chain as well as desaturation at the 12,13 linkage. Degradation to two-carbon units and resynthesis to linoleic acid is, of course, possible. A more likely conversion is the addition of a C_2 unit at the carboxyl end which would give 11-octadecenoic acid, a component found in considerable amount in *Asclepias* oil (1). The fact that 9-hexadecenoic and 11-octadecenoic acids occur together in quantity in *Asclepias* oil but do not so occur in other oils suggests that these two acids are formed in sequence.

There was some decrease in the total amount of octadecenoic acids (oleic + 11-octadecenoic) during the same period. This can be accounted for if it is assumed that the oleic acid was being converted to linoleic acid. Simmons and Quackenbush (7) showed a sequential formation of oleic and linoleic acids in the soybean seed by supplying radioactive sucrose to the plant, although they were not able to demonstrate a reduction in the level of labelled oleic acid. More recently, Yuan and Bloch have found that labelled oleic acid is converted to linoleic acid by yeast (8). Thus there is no doubt that this mode of biosynthesis of linoleic acid is possible and may take place in the developing seed.

Some of the results of the present work confirm or extend the conclusions of earlier workers. The sudden change in composition at a certain stage of seed development has been noted by Crombie in the palm kernel (5) and by Painter in flax seed (9). Formation of the greater part of the total oil in the first half of the period of development has been reported also by Eyre in flax seed (10) and by Humphries in the cocoa bean (11).

It is self-evident that the major fatty acid must form at a greater rate than the others. However, it was noted in the present work that this preferential increase took place at the middle stage of development. In the early and late stages the rate difference was less marked. Similar conclusions can be drawn from Grindley's data for cottonseed (3). The low content of free fatty acid throughout shows that there is no accumulation of free acids followed by later esterification, unless this occurred very shortly after flowering. Crombie found no evidence of such a sequence (5).

On the basis of the present and earlier work, therefore, the general process of oil synthesis in the seed appears to be as follows: (*a*) little oil is formed in the very early period of development and this oil differs in composition from the final product; (*b*) oil is formed rapidly at a middle stage and changes suddenly in composition to take on the fatty acid composition characteristic of the species; (*c*) oil continues to form to maturity, but more slowly, and the composition does not change materially; (*d*) the mechanism of fatty acid formation is not yet clear but probably includes conversion of unsaturated acids initially produced to acids of greater unsaturation or greater chain length, or both.

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THE EFFECT OF CERTAIN SULPHONYLUREA DRUGS ON PANTOTHENIC ACID METABOLISM IN THE RAT[1]

E. J. MIDDLETON AND J. M. MCLAUGHLAN

Abstract

Small amounts of chlorpropamide in the diet (125 mg/kg) did not affect the growth response of rats given pantothenic acid deficient or normal diets. The growth-retarding effect of 1000 mg chlorpropamide/kg of diet was not overcome by the addition of excess pantothenic acid to the diet. The hypoglycemic responses of normal and pantothenic acid deficient animals to insulin were similar, but the effect of tolbutamide on blood sugar levels was significantly less in pantothenic acid deficient animals than in normal controls. The acetylation of an intraperitoneally administered dose of sulphanilamide was significantly lowered when normal rats were given a concomitant oral dose of tolbutamide or propylthiouracil, but was unaffected by phenformin or insulin. Although pantothenic acid deficient animals acetylated less sulphanilamide than normal animals, the percentage of acetylation was not reduced further by treatment with tolbutamide.

It was concluded that tolbutamide and chlorpropamide apparently influence certain aspects of pantothenic acid metabolism in the rat, but is unlikely to influence significantly the over-all requirements for the vitamin.

Introduction

Apparent antivitamin activity has been observed clinically after prolonged administration of certain drugs. There is evidence that Isoniazid (1) and penicillamine (2) interfere with pyridoxine metabolism, and that primidone induces a folic acid deficiency in some patients (3). The possibility that additional drug–vitamin interrelationships may exist led McLaughlan, Shenoy, and Campbell (4) to propose rapid microbiological screening methods for the detection of antivitamin effects of drugs. In studies involving more than 50 drugs they found that only propylthiouracil and four sulphonylurea compounds interfered with pantothenic acid metabolism in the protozoon, *Tetrahymena pyriformis*.

In studies on the mode of action of tolbutamide, Boshnell, Zahnd, and Renold (5) found that the drug inhibited hepatic ketogenesis and suggested that it influenced coenzyme A metabolism. Other studies reported by Wrenshall (6) indicated that tolbutamide and carbutamide inhibited the in vitro acetylation of *p*-nitroaniline, a reaction requiring coenzyme A. Since coenzyme A contains pantothenic acid, it became of practical importance to investigate the effect of the sulphonylurea drugs on pantothenic acid metabolism. This paper describes a study of the effect of certain sulphonylurea drugs on pantothenic acid nutrition in the rat with respect to growth, acetylation of sulphanilamide, and hypoglycemic response.

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Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario.

Method

Sixty male weanling rats of the Food and Drug strain were fed for 2 weeks on a basal diet containing, in %, casein, 20; cornstarch, 60; corn oil, 10; salts, 4; alphacel, 5; and vitamin mixture, 1. The vitamin mixture used was that of Chapman *et al.* (7) without calcium pantothenate. The rats were then divided into three comparable groups of 20 rats each and given the basal diet alone or supplemented with 0.7 or 15.0 mg calcium pantothenate per 100 g of diet. Ten animals from each group also received 125 mg chlorpropamide per kg diet. In a second growth experiment, the level of drug was increased to 1000 mg per kg diet. Food consumption and body weight were recorded at weekly intervals for 6 weeks.

At the end of the first growth study we determined the effect, on the acetylation ability of the animals, of a sulphonylurea drug administered orally. Studies were also conducted on the effects of oral administration of propylthiouracil and phenformin, and of intramuscular injection of insulin, on the acetylation ability of rats receiving diets containing 0.7 and 15.0 mg calcium pantothenate per 100 g of diet without added chlorpropamide. The drugs were administered at the following concentration per kilogram body weight: tolbutamide, 200 mg; propylthiouracil, 200 mg; phenformin, 50 mg; and insulin, 1.0 unit. The animals were fasted for 24 hours (water supplied) and given an intraperitoneal injection of sulphanilamide (250 mg per kg body weight) and concomitant doses of the various compounds tested. The animals were then placed in metabolism cages, with free access to water but not food, and urine was collected for 24 hours. Free and total sulphanilamides in urine were determined by the method of Bratton and Marshall (8). Except for the days when they were on test, the animals continued to receive the diets given to them during the growth study.

The blood sugar levels of the animals given tolbutamide and insulin were determined at various time intervals after dosing, using the method of Nelson (9). Because of the findings with tolbutamide, the test with this compound was carried out twice.

Results and Discussion

Effect on Growth

Curves of weight gain of the animals fed the various dietary regimens for a 6-week experimental period are shown in Fig. 1. In agreement with previous studies by other workers (10) the animals fed a diet devoid of pantothenate gained weight at a significantly slower rate than those receiving 0.7 and 15.0 mg of the vitamin per 100 g of diet. Chlorpropamide at a level of 125 mg/kg diet had no significant effect on weight gain. It is known that this level is the maximum which can be added to the diet without producing growth depression (11) and might be expected to depress weight gain of animals given suboptimal amounts of pantothenate, if chlorpropamide interfered with pantothenate metabolism. In a second experiment, the drug was fed at 1000 mg/kg diet and

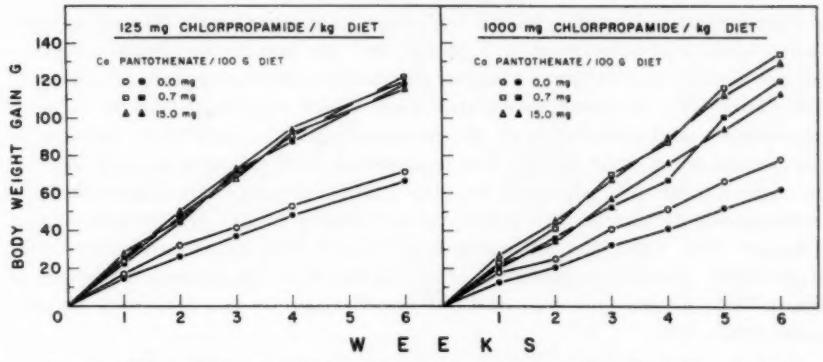

FIG. 1. Effect of chlorpropamide on the growth of rats fed various levels of pantothenic acid. Open symbols, diets without drug; closed symbols, diets with drug.

significantly depressed weight gain of animals given 0, 0.7, and 15 mg pantothenate/100 g diet. Excess pantothenate (15 mg/100 g) had no effect on chlorpropamide-induced weight retardation, thus it is not possible to draw conclusions that the growth-retarding effect of the drug is due to interference with pantothenic acid metabolism. However, it might be concluded that chlorpropamide had no significant effect on pantothenate requirement for growth.

Effect on Acetylation Reaction

Table I summarizes the acetylation data on each of the dietary regimens. In agreement with the findings of previous workers (12, 13) the percentage of acetylation of sulphanilamide (percentage of total urinary sulphanilamide in bound form) was lowered significantly in animals receiving diets with no added pantothenate. A concomitant oral dose of tolbutamide significantly decreased the acetylation ability of rats receiving adequate pantothenate, but had no effect in pantothenate-deficient animals. Inclusion of chlorpropamide in the diet for 6 weeks did not significantly alter the effects of tolbutamide on the acetylation ability of the rat.

TABLE I

Effect of tolbutamide and dietary calcium pantothenate on the acetylation ability of the rat

Chlorpropamide (mg/kg diet)	Level of calcium pantothenate (mg/100 g diet)	No. of animals	Acetylation	
			Tolbutamide (%)	Control (%)
—	0.0	9	30.6±0.6	33.7±1.2*
—	0.7	9	36.5±0.8	41.4±1.0
—	15.0	10	36.9±0.7*	41.5±1.2
125	0.0	9	32.1±1.5	33.2±1.4
125	0.7	10	36.4±0.8	42.6±1.2
125	15.0	10	36.9±1.0	42.2±1.0

*Standard error of the mean.

Phenformin and insulin, known hypoglycemic agents which showed no anti-pantothenic acid activity with *T. pyriformis* (4), had no significant effect on the percentage acetylation of a dose of sulphanilamide in rats given an adequate diet (Table II). However, propylthiouracil, which was found to have an anti-pantothenic acid activity for *T. pyriformis*, produced a significant decrease in the percentage of acetylation. The pantothenic acid deficient animals showed an approximate 25% decrease in their ability to acetylate sulphanilamide. Tolbutamide did not further reduce the acetylation ability of pantothenic acid deficient rats. This was not surprising since it was shown previously that acetylation values in pantothenic acid deficient animals tended to plateau at about 75% of normal values and did not decrease as the deficiency became more severe (14).

TABLE II

Effect of various drugs on the acetylation ability of normal rats

Drug	No. of animals	Acetylation (%)
Control (water)	6	42.2±1.3*
Propylthiouracil	6	32.9±1.6
Phenformin	6	39.7±1.6
Insulin	6	42.3±2.9

*Standard error of the mean.

Since the percentage of acetylation of sulphanilamide can be related to the state of pantothenic acid nutrition it would appear that the inhibitory effect exhibited by tolbutamide was probably due to interference with pantothenic acid – coenzyme A metabolism. Wrenshall (6) showed previously that the acetylation of *p*-nitroaniline in a soluble liver enzyme system with added coenzyme A was inhibited by the addition of tolbutamide or carbutamide to the medium.

Effect on Hypoglycemic Response

The data on the effect of dietary levels of pantothenate on the hypoglycemic response of rats to tolbutamide are summarized in Fig. 2.

The hypoglycemic response of the animals was maximal 4 hours after an oral dose of tolbutamide. The animals given the diet without added pantothenate showed a significantly decreased response to tolbutamide at 2 and 4 hours, as compared with those receiving diets supplemented with calcium pantothenate. This test was repeated with the same animals approximately two weeks later and a similar suboptimal response by the pantothenic acid deficient group was obtained. By comparison, insulin produced essentially the same hypoglycemic response in the three groups receiving the various levels of pantothenate. Winters *et al.* (15) reported an increased sensitivity to insulin in pantothenic acid deficient animals. The reason for the discrepancy between the present findings and those of Winters *et al.* is not apparent, but may be

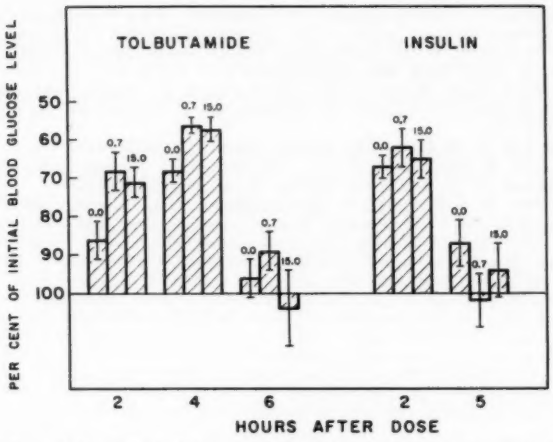

FIG. 2. Effect of the oral administration of tolbutamide or insulin on the hypoglycemic response of rats fed various levels of pantothenic acid. Numerals above each bar indicate milligrams of calcium pantothenate/100 g diet.

related to the severity of the pantothenic acid deficiency produced. Propylthiouracil was reported to have an antidiabetic effect (16); but unpublished work in our laboratory indicated that this drug did not have a direct hypoglycemic response similar to tolbutamide.

Although chlorpropamide had no effect on the pantothenic acid requirement of rats for growth, the acetylation and blood sugar studies indicated that tolbutamide might interfere with some aspects of pantothenic acid metabolism. It was of particular interest that both tolbutamide and propylthiouracil, which appeared to have antipantothenate activity for *T. pyriformis*, inhibited acetylation in the rat. These results indicate that there is good correspondence between the microbiological screening procedure with *T. pyriformis* (4) and the effect of these drugs on the acetylation of sulphanilamide in the rat. From a practical point of view it is evident that chlorpropamide is unlikely to produce a significant effect on the requirement for pantothenic acid and except in an extreme deficiency the state of pantothenic acid nutrition would have little if any influence on the hypoglycemic action of tolbutamide.

Acknowledgments

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SOME ENZYMIC PROPERTIES OF A PARTIALLY PURIFIED AMINOACYLASE OBTAINED FROM A POLISH VARIETY OF RAPESEED (BRASSICA CAMPESTRIS L.)[1]

K. OZAKI[2] AND L. R. WETTER

Abstract

Aminoacylase, which hydrolyzes N-acetyl amino acids, has been demonstrated in rapeseed. The enzyme was purified 150-fold by fractionation with ammonium sulphate and calcium phosphate gel. The purified preparation hydrolyzed N-acetyl amino acids and in addition certain dipeptides and chloracetyl-L-tyrosine. The enzyme was stable at −20° C and had a wide pH optimum (7.2 to 8.8). Cobalt ion was found to be an activator, while sulphydryl-reacting agents such as *p*-chloromercuribenzoate and some metal-chelating agents inhibited the hydrolysis. The enzyme showed rigorous specificity for the L-isomer. A comparison of the ratio of activities obtained for different enzyme preparations indicates that more than one enzyme is concerned in the hydrolysis of the different substrates.

Introduction

The presence of aminoacylases have now been well established in higher plants (1, 2). Chibata and Tosa (1) found that the enzyme was present in various tissues of a considerable number of plants. Later, Ozaki and Wetter (2) reported that aminoacylase was also widely distributed in the seeds of higher plants. The latter investigators showed that a wide variety of N-acetyl amino acids were hydrolyzed.

The only purified preparations to date have been obtained from animal sources. Birnbaum *et al.* (3) fractionated hog kidney to obtain two different aminoacylases that were classified according to their substrate specificity. Aminoacylase I hydrolyzed a variety of N-acetyl amino acids while aminoacylase II attacked only N-acetyl aspartic acid. Recently enzymes from other sources have been concentrated to obtain active preparations, e.g. Fungi (4). Since rapeseed contains an active aminoacylase (2), this material was chosen for a more detailed investigation of this enzyme. This report therefore deals with the partial purification and a study of some of its properties.

Experimental

Material and Methods

Polish variety rapeseed (*Brassica campestris* L.) was employed as the enzyme source. The seeds were ground in cold acetone (−10° C) in a Waring blendor, filtered, washed free of oil, and then air-dried. N-Acetyl amino acids, dipep-

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tides, and other amino acid derivatives were commercial preparations purchased from various sources. The calcium phosphate gel used in the adsorption step was prepared according to the method of Keilin and Hartree (5).

The enzymatic hydrolysis of N-acetyl amino acids was followed by employing the ninhydrin colorimetric method of Moore and Stein (6). The hydrolysis of peptides and other substituted amino acids was determined by measuring the release of CO_2 by a method described by McConnell (7). Protein was estimated by the method of Lowry *et al.* (8) with crystalline bovine serum albumin serving as the standard.

Estimation of Enzyme Activity

Unless otherwise stated, the reaction mixture contained 0.25 μmole of substrate (with respect to L-isomer), 0.05 μmole of Co^{++}, 5 μmoles of phosphate buffer (pH 7.2), and enzyme all in a final volume of 0.1 ml. The enzyme was first preincubated with Co^{++} and phosphate buffer for 1 hour at 37° C, then the substrate was added and the mixture was incubated at 37° C for a given period of time, usually 1 hour. The reaction was arrested by adding exactly 1 ml of ninhydrin reagent and heating the mixture for 20 minutes in a boiling-water bath. The sample was cooled and diluted with a known portion (usually 5 ml) of *n*-propanol:water (1:1). The optical density of the resulting solution was determined in a colorimeter at 570 mμ and the free amino acid concentration was obtained from the appropriate standard curve. Blanks were determined for all assays. A unit of enzyme activity is expressed as the amount of enzyme that will hydrolyze 0.1 μmole of N-acetyl amino acid in 1 hour at 37° C under the conditions described.

The enzymic hydrolysis of peptides and amino acid derivatives other than N-acetyl was performed in 0.4 ml of solution containing 2.5 μmoles of substrate (L-isomer), 0.2 μmole of Co^{++}, 25 μmoles of phosphate buffer (pH 7.2), and enzyme. The enzyme preparations were preincubated with Co^{++} and phosphate for ½ hour and then incubated with substrates for either 1 or 2 hours at 37° C along with appropriate controls. The reaction was stopped by adding the acid–ninhydrin reagent and the amount of free amino acid released was determined as described by McConnell (7).

Partial Purification of Enzyme

All operations were carried out at 2° C and unless otherwise noted the buffer employed was 0.05 *M* phosphate (pH 7.2). Ground oil-free rapeseed (60 g) was homogenized in a "Vir-Tis" homogenizer for 5 minutes at 40,000 r.p.m. in 600 ml of cold phosphate buffer. The homogenate was centrifuged and the supernatant dialyzed overnight against phosphate buffer. This dialyzate is designated as "crude extract" in Table I.

One hundred and twenty milliliters of saturated ammonium sulphate (adjusted to pH 7) was slowly added to 480 ml of dialyzate with constant stirring. The 20% saturated (with respect to ammonium sulphate) solution was centrifuged and the residue was discarded. Sufficient saturated ammonium

TABLE I

Summary of the purification of aminoacylase* from rapeseed

Fraction	Volume (ml)	Total protein (mg)	Total units†	Specific activity	Yield (%)
Crude extract	480	10,300	10,460	1.01	100
Ammonium sulphate I	90	3,120	6,580	2.11	64
Gel treatment I	127	255	2,870	11.3	32
Gel treatment II	134	74	1,635	22.1	18
Ammonium sulphate II	10	4.3	653	152	7

*Enzyme activity was tested by using N-acetyl-L-leucine as the substrate.
†The units employed are described in the text.

sulphate (200 ml) was then added to give a solution that was 40% saturated. The precipitate obtained was dissolved in a minimum volume of phosphate buffer and dialyzed overnight against the same buffer (ammonium sulphate I).

One hundred milliliters of calcium phosphate gel (30 mg per ml) was added to every 100 ml of enzyme solution containing 3.47 g of protein. This mixture was stirred for 1 hour, centrifuged, and the precipitate discarded (gel treatment I). Additional gel (25 ml to every 100 ml of protein solution which now contained 26.5 mg of protein) was added to the supernatant and stirred for 1 hour. After centrifugation the residue was discarded and the supernatant (gel treatment II) which contained the enzyme was saved. The two-step gel treatment was employed because greater purification was obtained by this technique.

Saturated ammonium sulphate was added to the supernatant (from gel treatment II) and the precipitate obtained between 30 and 35% saturation was dissolved in a minimum volume of phosphate buffer. This solution was dialyzed against phosphate buffer overnight (ammonium sulphate II) and stored at −20° C. Table I summarizes the fractionation procedure when 60 g of ground oil-free rapeseed was employed. A 150-fold purification was obtained with an over-all yield of 7%.

Results

Effect of Time and Enzyme Concentration on Activity

The reaction rate for the purified enzyme was zero order (Fig. 4). The hydrolysis of N-acetyl amino acids was also directly proportional to the enzyme concentration as shown in Fig. 1. Complete hydrolysis of the substrate was obtained when the experimental conditions described above were employed.

The purified enzyme was stable for at least 1 month when stored at −20° C in 0.05 *M* phosphate buffer (pH 7.2). Therefore all purified samples were stored under these conditions.

Effect of pH

The pH optimum for the aminoacylase of rapeseed was broad as shown in Fig. 2. Maximum hydrolysis of both substrates was obtained from pH 7.2 to pH 8.8. There was no apparent effect of buffer salts on the activity.

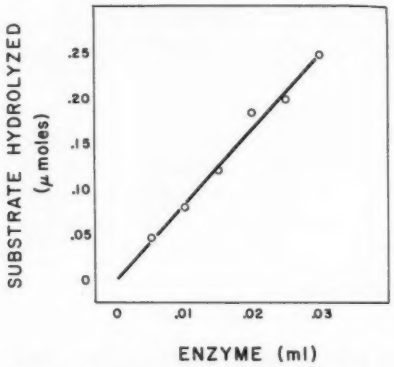

FIG. 1. The effect of enzyme concentration on the hydrolysis of N-acetyl-L-leucine. The assay was performed on 0.1 ml of reaction mixture, the color produced by ninhydrin was diluted with either 5, 10, or 15 ml of 50% *n*-propanol (v/v). The reaction mixture contained the various components in the quantities described in the text. The protein concentration was 4.24 μg per 0.01 ml of enzyme solution.

Effect of Temperature

The optimum temperature for the reaction was approximately 50° C (Fig. 3). An activation energy of 12,600 calories per mole was obtained when the hydrolysis rates obtained for the lower temperatures (7° C to 42° C) were employed for an Arrhenius plot. This value is similar to those found for enzyme reactions in which hydrolysis is involved. The slope for the higher temperatures changed and the transition temperature occurred at about 40° C but at the present time there is no explanation for this.

Effect of Divalent Cations

It was observed that purification of the enzyme resulted in a marked decrease in activity. Since Smith had observed that glycylglycine dipeptidase required Co^{++} (9) and leucine aminopeptidase required either Mn^{++} or Mg^{++} (10) for maximum activity, a loss of divalent cations was suspected as causing the loss of activity. Cobalt is the best cation activator of the seven tested (Table II). The effect of cations on the activity was similar when N-acetyl-L-leucine and N-acetyl-DL-phenylalanine were employed. Ferrous and zinc ions caused the next greatest response but this was much less than the response obtained with Co^{++}.

An interesting observation concerning the effect of Co^{++} on enzyme activity is illustrated in Fig. 4. The cation activation of the enzyme is not instantaneous, but is comparatively slow. For the conditions used in this study approximately 20 minutes was required to achieve maximum activation and for this reason the purified enzyme was preincubated with Co^{++} before the assay was carried out. This is in marked contrast to the report by Marshall *et al.* (11), who observed that renal acylase I was very rapidly activated by Co^{++} under their conditions. Cobalt exerts its effect primarily on the enzyme and not the sub-

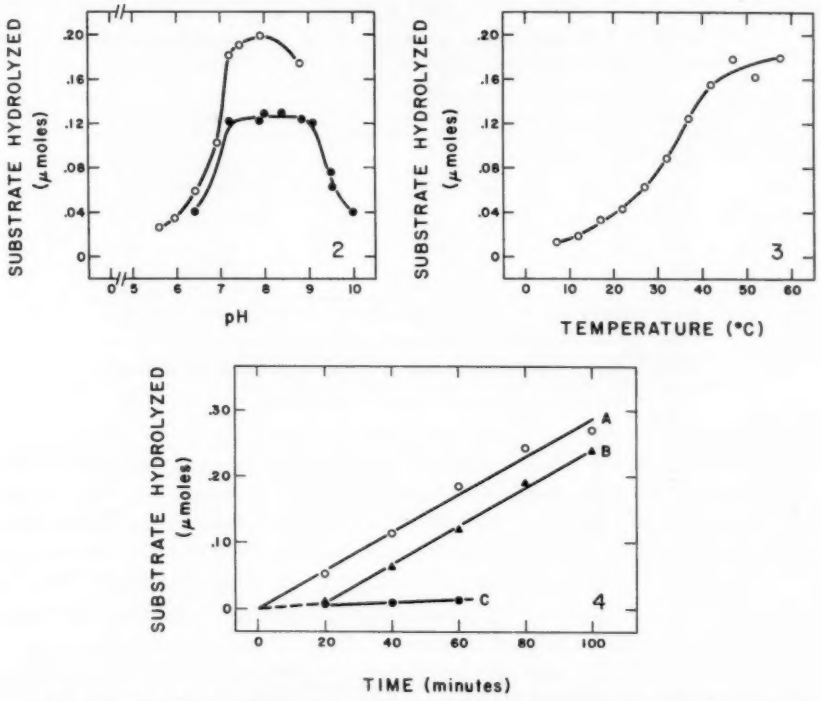

FIG. 2. The effect of pH on aminoacylase activity. The experimental conditions were the same as described in the text except for variations in pH. Phosphate buffers were employed for the pH range from 5.60 to 7.9, borate buffers from pH 8.00 to 8.85, and carbonate buffers from pH 9.0 to 10.0. ○——○ N-Acetyl-L-leucine was the substrate and each assay contained 8.5 μg of protein. ●——● N-Acetyl-DL-phenylalanine was the substrate and the protein concentration was 1.7 μg per assay.

FIG. 3. The effect of temperature on enzyme activity. The assay was set up as described in the text. N-Acetyl-DL-phenylalanine was used for the substrate and the enzyme concentration was 1.7 μg of protein per assay.

FIG. 4. The effect of time and Co^{++} on the hydrolysis of N-acetyl-L-leucine by aminoacylase. For color development 0.1 ml of reaction mixture was withdrawn at various times from 1 ml of reaction mixture containing 2.5 μmoles of substrate (with respect to L-isomer), 0.5 μmole of Co^{++}, 25 μmoles of phosphate buffer, and enzyme. (A) The enzyme was preincubated with Co^{++} and buffer for 1 hour at 37° C before the substrate was added. (B) The substrate was preincubated with Co^{++} and phosphate as described in A before the enzyme was added. (C) No Co^{++} was used.

strate, as prior incubation of the substrate with the cation did not result in maximum activity. A similar observation was made by Mohamed and Greenberg (12) in their report on arginase.

The effect of Co^{++} concentration is shown in Fig. 5 and maximum activity is obtained at a concentration of 0.05 μmole. The curve for the purified preparation can be plotted as described by Lineweaver and Burk (13). The K_m for Co^{++} obtained from this plot is 1.53×10^{-4} M for the conditions of the assay.

TABLE II

The effect of divalent cations on the activity of rapeseed aminoacylase

Additions	Relative activity (%)	
	N-Acetyl-L-leucine	N-Acetyl-DL-phenylalanine
None	2	4
Co^{++}	100*	100†
Ca^{++}	16	6
Fe^{++}	22	10
Mg^{++}	14	0
Mn^{++}	6	0
Ni^{++}	10	0
Zn^{++}	18	18

NOTE: The assay was carried out as described in the text, except that 0.01 μmole of divalent ions per assay was employed.

*100% activity corresponds to 0.05 μmole of N-acetyl-L-leucine hydrolyzed in 1 hour by 2.3 μg of protein.

†100% activity corresponds to 0.12 μmole of N-acetyl-DL-phenylalanine hydrolyzed in 1 hour by 0.8 μg of protein.

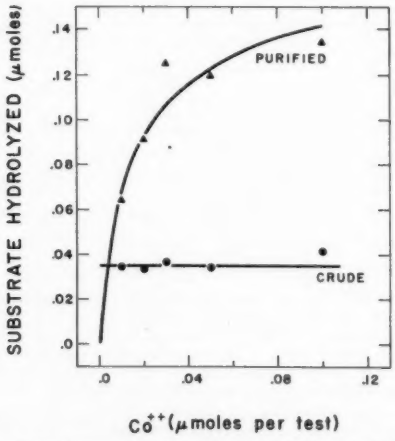

FIG. 5. The effect of Co^{++} concentration on the hydrolysis of N-acetyl-L-leucine. The test was carried out as described in the text except that the Co^{++} concentration was varied.

Effect of Inhibitors

Table III summarizes the results obtained when sulphydryl-blocking compounds and metal-complexing agents were added to the enzyme mixture. N-Acetyl-L-leucine was employed as the substrate for this experiment. *p*-Chloromercuribenzoate is the most effective inhibitor of the sulphydryl-blocking compounds, followed by *o*-iodosobenzoate. The two thioalkylating reagents, iodoacetate and N-ethylmaleimide, are much poorer inhibitors as might be expected. The inhibition produced by *p*-chloromercuribenzoate was completely reversed by 3 times the concentration of glutathione and the order in which the two materials were added had no effect on the hydrolysis.

Since the enzyme requires Co^{++} for maximum activity, one would expect

TABLE III

The effect of various compounds on the activity of aminoacylase

Compound	Concentration* (μmoles)	Relative activity (%)
None	—	100
p-Chloromercuribenzoate	0.003	37
o-Iodosobenzoate	0.005	60
Iodoacetate	0.1	80
N-Ethylmaleimide	0.3	103
Versene	1.0	4
α,α'-Dipyridyl	0.2	11
o-Phenanthroline	0.1	59
Sodium citrate	1.0	68
8-Hydroxyquinoline	0.1	112

NOTE: The incubation was carried out as follows: The enzyme, buffer, and Co^{++} were incubated for 40 minutes at 37° C, then the inhibitor was added and incubated for another 20 minutes, finally the substrate was added.

*The concentration designates the amount of material added to the 0.1 ml used for the assay.

metal-complexing reagents to have an inhibitory effect. Table III shows that this is the case and that versene (disodium ethylenediaminetetraacetate) is most effective in removing Co^{++} from the enzyme. α,α'-Dipyridyl is as effective as versene while the others are not good complexing agents. This is particularly true of 8-hydroxyquinoline.

Effect of Hydrolysis Products and D-*Isomers*

In a separate experiment in which both the D- and L-isomers were used as substrates only the L-isomer was hydrolyzed. Since many substrates can only be obtained as DL-mixtures one might expect an inhibitory effect from the D-isomer but this isomer at twice the concentration has no effect on the hydrolysis of the L-isomer (Table IV).

TABLE IV

The effect of hydrolytic products and D-isomers on the enzyme activity

Addition (μmoles)		Relative activity* (%)
None		100
L-Phenylalanine	0.25	101
	0.50	101
Sodium acetate	0.25	99
	0.50	98
N-Acetyl-D-phenylalanine	0.25	95
	0.50	104

*N-Acetyl-DL-phenylalanine was employed as the substrate and 100% activity corresponds to 1.5 units of enzyme. To test the effect of the D-isomer, the L-isomer was employed as the substrate.

The results in Table IV also show that the products of the reaction do not affect the hydrolysis of the substrate. Thus one would expect that the reaction is not reversible under the conditions employed.

Specificity of the Enzyme

The data in Table V show that only some of the acetylated amino acids were hydrolyzed. The two most readily hydrolyzed were N-acetyl phenylalanine and tyrosine, while the other aromatic amino acid, tryptophan, was almost

TABLE V

A comparison of the hydrolysis of different N-acetyl amino acids by purified rapeseed aminoacylase

Compound	Relative activity* (%)
Experiment I	
N-Acetyl-L-leucine	100
N-Acetyl-DL-phenylalanine	480
N-Acetyl-DL-tyrosine	460
N-Acetyl-DL-methionine	230
Experiment II	
N-Acetyl-L-leucine	100
N-Acetylglycine	0
N-Acetyl-DL-alanine	6
N-Acetyl-DL-glutamic acid	0
N-Acetyl-DL-valine	11
N-Acetyl-DL-tryptophan	10
N-Acetyl-DL-proline†	0

*The assay was carried out as described in the text except for the substrate employed. The enzyme concentration was varied as follows: 0.85 μg of protein was used per assay for experiment I and 4.25 μg of protein was employed per assay for experiment II.

†The color produced by the addition of ninhydrin was read at 440 mμ.

completely inactive. The next most active substrate was N-acetyl methionine followed by acetylated leucine. Other N-acetyl amino acids were hydrolyzed to some extent but only slightly when compared with the more active substrates. The data in Table V illustrate the importance of the amino acid residue which is particularly well demonstrated when the N-acetylated amino acids of leucine, valine, alanine, and glycine are compared. The removal of one methylene group from leucine reduces the activity by a factor of 10. The elimination of another carbon atom decreases the activity still further until the ability to hydrolyze these compounds is completely lost as illustrated by N-acetylglycine.

Since aminoacylase hydrolyzes the peptide bond one would expect that certain dipeptides and substituted amino acids might also be hydrolyzed (3). The results obtained for this investigation are shown in Table VI. The hydrolysis values were obtained by employing a different assay method as described in the section on Materials and Methods. Since the crude mixture does not hydrolyze either benzoyl or carbobenzoxy amino acids, one must conclude that there is no carboxypeptidase present in rapeseed extracts. The purified enzyme preparation hydrolyzes several different dipeptides which are related to the N-acetyl substrates in that the terminal amino acids are the same. No comment can be made at present as to whether the same enzyme is responsible for the

hydrolysis of these different substrates. A comparison of activity ratios found for the crude preparation and the purified fraction when glycyl-DL-leucine, L-alanyl-L-leucine, glycyl-DL-phenylalanine, and chloroacetyl-L-tyrosine were used as substrates suggests that perhaps three different enzymes are responsible for their hydrolysis. Unfortunately the hydrolysis rate of the dipeptide, glycyl-DL-leucine, cannot be directly compared with the hydrolysis rate of N-acetyl-DL-leucine. However, some qualitative comparisons indicate that different enzymes are involved as N-acetyl-DL-phenylalanine is hydrolyzed 7 times more rapidly than N-acetyl-L-leucine (Table V); on the other hand, glycyl-L-phenylalanine is hydrolyzed more slowly than is glycyl-DL-leucine.

There are enzymes present in the crude extract that are completely lost by the fractionation procedure employed. There is also further demonstration of the complete isomeric specificity of the amino residue of the dipeptide (L-leucyl-L-tyrosine and D-leucyl-L-tyrosine (Table VI)).

TABLE VI

Relative rates of hydrolysis of various peptides and substituted amino acids by rapeseed enzyme preparations

Substrate	Relative activity (%)	
	Crude	Purified
Benzoyl-DL-leucine	0	N.T.*
N-CB†-DL-leucine	0	N.T.
Glycyl-DL-leucine	100	100
L-Alanyl-L-leucine	74	32
N-CB-Glycyl-DL-phenylalanine	0	N.T.
Glycyl-L-phenylalanine	65	62
L-Leucyl-L-phenylalanine	57	0
L-Phenylalanyl-L-phenylalanine	119	0
CB-α-L-Glutamyl-L-tyrosine	0	N.T.
Chloroacetyl-L-tyrosine	60	118
L-Leucyl-L-tyrosine	64	0
D-Leucyl-L-tyrosine	0	N.T.
L-Leucylglycylglycine	44	0

NOTE: The assay was carried out as described in the text. The value of 100 for glycyl-DL-leucine represents that enzyme concentration which resulted in 60% hydrolysis of the substrate.

*N.T. means that these materials were not tested for this enzyme preparation.

†CB designates the carbobenzoxy group.

A comparison of ratios of activity for different enzyme preparations sometimes gives an indication as to whether one or more enzymes are involved. Table VII presents ratios for the four most readily hydrolyzed acetylated amino acids for the crude extract and the purified preparation. The results suggest that there may be two different enzymes, one that hydrolyzes N-acetyl-DL-methionine and one that hydrolyzes the other three.

Discussion

The properties of partially purified rapeseed aminoacylase are similar in many respects to an enzyme (acylase I) found in animal tissue. Enzymes from both

sources are stable over relatively long periods of time. The pH optimum is also similar for both preparations while the effect of temperature is quite different; acylase I is more stable at higher temperatures. Both enzymes require a divalent cation as an activator and in this respect both are like other peptide-hydrolyzing enzymes (10, 11, 14, 15). Rapeseed aminoacylase appears to act only as an hydrolyzing enzyme thus differing from acylase I. Recently Russian workers (16, 17) have reported that acylase I can be employed to synthesize acetylated amino acids. The differences in experimental conditions could account for the different results that were obtained when the Russian work (16, 17) and our work are compared.

The specificity of the enzyme is not absolute and since the work was done with partially purified preparations one might expect to find lack of specificity. From Table V it is seen that at least four acetylated amino acids are hydrolyzed by the preparation; those having the aromatic residue are most readily attacked, followed by methionine and leucine. Other workers (18) have observed the same lack of specificity for acylase isolated from other sources and have concluded that one enzyme is responsible for the hydrolysis. However, there is some evidence in this work to suggest that there are two enzymes concerned; the ratios shown in Table VII indicate that perhaps another enzyme is hydrolyzing N-acetyl-DL-methionine.

TABLE VII

A comparison of the activity of different substrates with different enzyme preparations

Substrate	Ratio* of activity	
	Crude	Purified
N-Acetyl-L-leucine	1	1
N-Acetyl-DL-phenylalanine	4.1	3.8
N-Acetyl-DL-tyrosine	4.3	4.2
N-Acetyl-DL-methionine	3.4	2.2

NOTE: The enzyme concentration was adjusted so that both preparations resulted in 15% hydrolysis of N-acetyl-L-leucine.
*The ratio was obtained by comparing hydrolysis rates for different substrates to N-acetyl-L-leucine.

The aminoacylase from rapeseed also hydrolyzed several dipeptides as shown in Table VI. Whether this is due to one or more enzymes is not known but once again this lack of specificity has been observed for other acylases (3, 18). It would appear that one enzyme is responsible for the hydrolysis of glycyl-DL-leucine and glycyl-L-phenylalanine while different enzymes are responsible for the breakdown of L-alanyl-L-leucine and chloroacetyl-L-tyrosine. Since the preparation used in this investigation was of unknown purity, the question as to whether one or more enzymes is involved cannot be resolved.

It will be seen from Table VI that rapeseed is a rich source of enzymes that hydrolyze a large variety of dipeptides and substituted amino acids. The crude extract contains enzymes that attack amino acid derivatives and these are

completely removed by the fractionation technique employed to obtain aminoacylase. One might note that the crude extract has the ability to hydrolyze both L-phenylalanyl-L-phenylalanine and L-leucyl-L-phenylalanine. Rapeseed also has an enzyme which hydrolyzes the tripeptide L-leucylglycylglycine.

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VITAMIN A DEFICIENCY AND ISOPRENOID METABOLISM[1]

W. E. J. PHILLIPS

Abstract

Vitamin A deficiency in the intact rat evokes an increase in liver ubiquinone and the incorporation of mevalonate into ubiquinone and squalene. This aberration is caused by a metabolic block between squalene and cholesterol. The addition of vitamin A in vitro to vitamin A deficient liver homogenates does not influence the metabolic defect. The vitamin A deficient guinea pig does not exhibit an increase in liver ubiquinone or incorporation of mevalonate into ubiquinone or squalene, but the incorporation into sterols is enhanced.

Introduction

Lowe, Morton, and Harrison (1) observed in vitamin A deficient rat liver increases in two substances tentatively designated as SA and SC. These substances are now known as ubiquinone (2) and ubichromenol (3) respectively. Morton and Phillips (4) confirmed this increase and showed that the ubiquinone content of rat liver increases steadily as the deficiency syndrome develops. Many experimental approaches to explain the mechanism of the deranged metabolism were unsuccessful (5–7).

The isoprenoid side chain of ubiquinone and ubichromenol bears a structural relationship to endogenous sterols. Gloor and Wiss (8, 9) have recently shown that the rate of incorporation of mevalonic acid into ubiquinone, squalene, and sterols is dependent on the nutritional supply of vitamin A. It has been suggested that an interaction between vitamin A and ubiquinone takes place at a metabolic level. Experiments are described in this paper which demonstrate that the effects of vitamin A deficiency observed in the rat are not similar in all species. In all probability there is no direct interaction between vitamin A and ubiquinone metabolism. It is suggested that the effect is mediated by a metabolic block in cholesterol biosynthesis.

Experimental

General

Animals, male rats of the Wistar strain, were placed at weaning on a vitamin A deficient diet:

casein (vitamin free)	18%,
dried brewer's yeast	8%,
sugar	65%,
cottonseed oil*	5%,
salt mix, U.S.P. XIV	4%.

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Contribution No. 59 from the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Canada.

*The cottonseed oil contained per 100 g: vitamin E as *dl*-α-tocopheryl acetate, 60 mg; vitamin K as 2-methyl-1,4-naphthoquinone, 1.5 mg; vitamin D_2, 8000 units; or this diet supplemented with 5000 I.U. of stabilized vitamin A acetate per kilogram of diet.

The guinea pigs were litter-mate males fed a vitamin A deficient diet, which was basically extracted soybean meal supplemented with minerals and vitamins excluding vitamin A. Ascorbic acid was administered hypodermically once weekly at the rate of 5 mg per animal. One animal received orally 1500 I.U. vitamin A acetate per week.

Saponification, Chromatography, and Radioactivity Measurements

These methods have been described previously (10).

Labelled Precursors

2-C^{14}-Mevalonic acid was prepared by hydrolysis of the dibenzylethylenediamine salt purchased from Volk Radio-Chemical Co., Chicago, Illinois. The compound was dissolved in aqueous solution and adjusted to pH 7.0. The tracer dose was administered by intraperitoneal injection.

Liver In Vitro System

The liver homogenate preparation, the incubation procedure, and the technique for measuring the incorporation of mevalonate into cholesterol have been described by Migicovsky (11) and Migicovsky and Wood (12).

(a) Incorporation of Mevalonic Acid into Liver Non-saponifiable Constituents of a Normal and Vitamin A Deficient Rat

The vitamin A deficient rat used in this experiment was fed the vitamin A deficient diet for a period of 62 days. At the time of injection of the mevalonic acid it exhibited severe symptoms of the deficiency syndrome including xerophthalmic lesions and muscular inco-ordination. This animal and the control vitamin A supplemented rat were killed 24 hours after the administration of the tracer dose. Chromatography of the liver non-saponifiable material was accomplished by elution of the constituents from 10 g of acid-washed neutralized alumina partially deactivated to Brockmann grade 3. Twenty-one fractions of 25 ml each were collected using the following eluents: 50 ml of petroleum ether (P), 50 ml 2% diethyl ether (E) in P, 50 ml 4% E in P, 100 ml 6% E in P, 50 ml 8% E in P, 150 ml 20% E in P, and 75 ml E. The ultraviolet absorption spectrum and the amount of radioactivity were determined in each fraction. The distribution of the radioactivity and the total amount of ubiquinone in each fraction are shown in Fig. 1. Fractions 1 and 2 which have been designated peak 1 represent the tissue hydrocarbons including squalene. Fractions 5 to 9 inclusive designated peak 2 represent the ubiquinone component while fractions 10 to 16 designated peak 3 represent the total sterol. Total ubiquinone was calculated from the ultraviolet absorption and is expressed as ubiquinone (50). The experimental design and some observations are shown in Table I.

Comparison of absolute incorporation of mevalonic acid into the various non-saponifiable constituents is of doubtful value due to the probable influence of body weight difference resulting from the limitation of growth imposed by vitamin A deficiency. The percentage distribution of the radioactivity

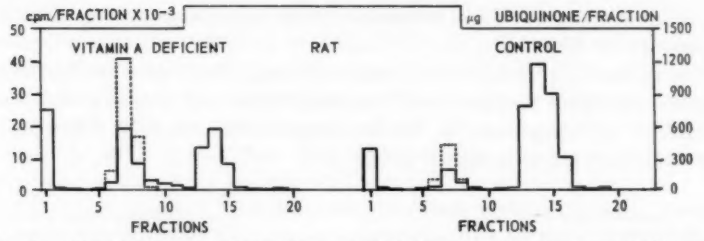

FIG. 1. Distribution of radioactivity and ubiquinone in the non-saponifiable matter from liver tissue of normal and vitamin A deficient rats injected with 2-C^{14}-mevalonic acid. —— c.p.m./fraction, – – – μg ubiquinone/fraction.

TABLE I

Incorporation of 2-C^{14}-mevalonic acid into non-saponifiable constituents of rat liver

Treatment	Vitamin A supplemented	Vitamin A deficient
Body wt. (g)	162	84
Liver wt. (g)	7.89	3.13
% non-saponifiable material	0.432	0.797
Dose μmoles mevalonic acid	8.43	8.43
c.p.m. in dose	8,976,000	8,976,000
μmoles incorporated × 10^3 into non-saponifiable material	130	109
% of dose incorporated	1.5	1.3
	μmoles mevalonic acid incorporated × 10^3	
Peak I (hydrocarbon)	11.4 (10.3)*	24.8 (22.8)
Peak II (ubiquinone)	11.5 (8.8)	32.5 (29.8)
Peak III (sterols)	103.5 (79.5)	46.6 (42.7)
μg ubiquinone (total)	647	1947
μg ubiquinone/g liver	82	622

*Peak expressed as a percentage of the incorporation in the total non-saponifiable material.

within the non-saponifiable material is, however, significantly altered by vitamin A deficiency. The synthesis of sterols as determined from the incorporation of mevalonic acid is limited while the hydrocarbon fraction (squalene) and ubiquinone have accumulated in the deficiency state. The radioactivity in the sterol fraction, expressed as a percentage of that in the total non-saponifiable fraction, is reduced from 80% to 43% by vitamin A deficiency. The demonstration of a metabolic block is a reproducible effect as shown by the work of Gloor and Wiss (8, 9) and further experimentation in this laboratory. The effect, however, is modified by the stage of the vitamin A deficiency syndrome. In two experiments conducted with vitamin A deficient animals at the beginning of the growth plateau stage, the percentage of radioactivity incorporated into the sterol fraction and expressed as a percentage of the incorporation in the total non-saponifiable material, 4 hours after a tracer dose of mevalonic acid, was reduced in one case from 87 to 79% and in the other from 82 to 74%.

(b) Incorporation of 2-C¹⁴-Mevalonic Acid into Guinea Pig Liver Non-saponifiable Material

The experimental design and procedures employed were similar to those previously described for the rats. The distribution of radioactivity and the total amount of ubiquinone in the non-saponifiable material following chromatographic separation is shown in Fig. 2.

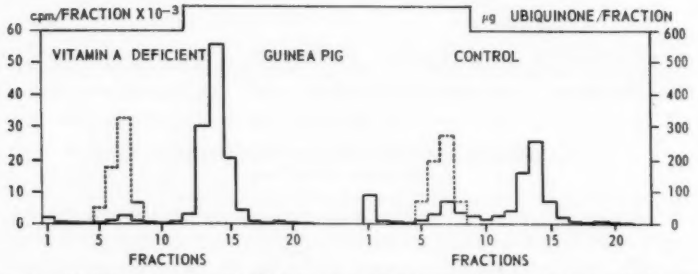

FIG. 2. Distribution of radioactivity and ubiquinone in the non-saponifiable matter from liver tissue of normal and vitamin A deficient guinea pigs injected with 2-C¹⁴-mevalonic acid. —— c.p.m./fraction, – – – μg ubiquinone/fraction.

The experimental results are shown in Table II. The percentage of the dose of mevalonic acid incorporated into the non-saponifiable constituents of guinea pig liver was similar to that observed for rats. The effect of vitamin A deficiency on the distribution of radioactivity between the main components in the gross non-saponifiable material demonstrated a significant species difference. Vitamin A deficiency in the guinea pig increased the incorporation of mevalonic acid into the sterol fraction and decreased the incorporation into

TABLE II

Incorporation of 2-C¹⁴-mevalonic acid into non-saponifiable constituents of guinea pig liver

Treatment	Vitamin A supplemented	Vitamin A deficient
Body wt. (g)	289	232
Liver wt. (g)	13.0	14.6
% non-saponifiable material	0.411	0.370
Dose μmoles mevalonic acid	9.92	9.92
c.p.m. in dose	10,560,000	10,560,000
μmoles incorporated × 10³ into non-saponifiable material	91	121
% of dose incorporated	0.92	1.22
	μmoles mevalonic acid incorporated × 10³	
Peak I (hydrocarbon)	8.6 (9.4)*	2.5 (2.1)
Peak II (ubiquinone)	14.3 (15.6)	5.7 (4.7)
Peak III (sterols)	56.3 (61.8)	110.1 (91.4)
μg ubiquinone (total)	606	625
μg ubiquinone/g liver	47	43

*Peak expressed as a percentage of the incorporation in the total non-saponifiable material.

the hydrocarbons (squalene) and ubiquinone. The influence of vitamin A deficiency on sterol metabolism in the guinea pig has been further confirmed by showing that 4 hours after a tracer dose of acetate-1-C^{14} the sterol fraction accounted for 45% of the radioactivity in the non-saponifiable material in the control animal while in the deficient animal the radioactivity in the sterol accounted for 69% of the total. The concentration of hepatic ubiquinone in all cases was unaltered by the stress of vitamin A deficiency.

(c) Effect of Vitamin A In Vitro on the Biosynthesis of Isoprenoid Compounds

To determine whether the derangement observed in sterol biosynthesis in the vitamin A deficient rat in vivo was a direct effect of the lack of vitamin A per se or a secondary effect of the deficiency syndrome, a number of in vitro experiments were conducted. Liver homogenates were prepared from vitamin A deficient rats. In the subsequent discussion the terms "deficient" and "normal" are used to denote homogenate fractions prepared from the livers of rats raised as described above.

The data shown in Table III are average values obtained from incubating 2 ml of liver homogenates. Each incubation flask contained 0.456 μmole of mevalonic acid-2-C^{14}. A vehicle for the addition of vitamin A was prepared by centrifuging the supernatant from a vitamin A deficient rat liver homogenate in a Spinco Model L centrifuge at 100,000$\times g$ for 30 minutes. The resulting supernatant fraction will be referred to as "supernate". Thirty milligrams of all-trans vitamin A alcohol was homogenized in 3 ml of supernate. Each incubation flask received 0.1 ml of either supernate or supernate containing vitamin A.

TABLE III

Effect of vitamin A on the incorporation of mevalonic acid in vitro into sterol by liver homogenates

Treatment	Addition to incubation mixture	Total mevalonic acid incorporated into sterols (μmoles$\times 10^3$)
Normal	Supernate	24.2 ($\pm$1.9)*
Normal	Supernate + vitamin A	26.1 ($\pm$1.3)
Deficient	Supernate	9.0 ($\pm$1.6)
Deficient	Supernate + vitamin A	7.2 ($\pm$2.1)

*Standard error of the mean.

It is evident that liver homogenates prepared from deficient animals are severely limited in sterol synthetic ability. The addition of vitamin A to the incubation mixture does not restore activity.

Discussion

In their search for the metabolic role of vitamin A many workers have directed their attention to sterol metabolism. Differences in experimental observations and conclusions have left a relationship, if any, in a state of

confusion. Collazo *et al.* (13) observed a lower cholesterol content in blood of vitamin A deficient rats as compared with normal rats while elevated cholesterol levels were reported by Ralli and Waterhouse (14). Green *et al.* (15), studying both serum and liver cholesterol levels, could detect no effect of vitamin A deficiency at any stage of the deficiency syndrome. They concluded that "in a major and quantitative sense cholesterol metabolism is not responsive to vitamin A depletion". The use of C^{14} in measuring cholesterol biosynthetic activity did not resolve the problem. Migicovsky (11) demonstrated the inability of liver homogenates from vitamin A deficient rats to synthesize cholesterol from C^{14}-acetate. On the other hand, Wolf *et al.* (16) concluded from their study of metabolism of acetate-1-C^{14} in intact animals that vitamin A is not required for cholesterol biosynthesis.

The results in this paper agree completely with the recent reports of Gloor and Wiss (8, 9) that in the intact vitamin A deficient rat a major metabolic block in the formation of cholesterol occurs after the formation of squalene. However, the postulate that vitamin A or a metabolic derivative is directly involved in the last steps of cholesterol synthesis does not appear to be correct. This effect of avitaminosis in the rat did not occur in another mammalian species, the guinea pig, and the addition of vitamin A in vitro to a vitamin A deficient rat liver homogenate did not restore cholesterol synthetic activity. It would appear that the limitation of cholesterol biosynthesis during vitamin A deficiency is a secondary effect of the syndrome. Vitamin A deficiency in the rat, although affecting the rate of cholesterol biosynthesis, does not alter tissue concentrations until the very terminal stages (4). The increase in mevalonate incorporation into the sterols of the vitamin A deficient guinea pig are interesting to note and may be a reflection of the relatively high percentage of "fast-reacting" sterols in the total sterol fraction.

Little is known of the complete biosynthesis of the ubiquinones, particularly the origin of the benzoquinone nucleus. It is reasonable, however, both from experimental data and structural formula considerations to postulate a biosynthetic path for the isoprenoid side chain. Figure 3 is essentially a simplified schematic diagram of the accepted pathway for the biosynthesis of sterols. It would appear that farnesyl pyrophosphate is the point of divergence between the cholesterol and ubiquinone pathways. Squalene is formed from a condensation of farnesyl pyrophosphate molecules yielding a hydrocarbon with a center of symmetry. Ubiquinone, on the other hand, has no center of symmetry (17).

Vitamin A deficiency has been shown to increase the concentration of liver ubiquinone in the rat (1, 4) but not in the guinea pig (2). The present work confirms these observations and demonstrates further that vitamin A deficiency in the guinea pig does not increase the incorporation of mevalonate into ubiquinone or squalene. It has been suggested (8) that in the rat an interaction between vitamin A and ubiquinone takes place. The present experiments strongly suggest that no such interaction takes place but that the increase in

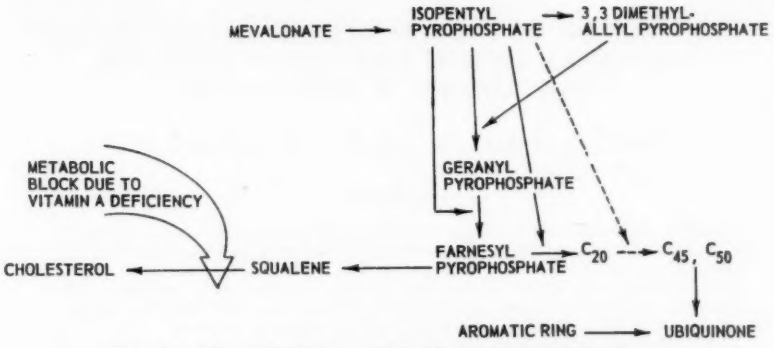

FIG. 3. Schematic diagram of the biosynthesis of ubiquinone.

hepatic ubiquinone in the rat is a result of vitamin A deficiency suppressing cholesterol biosynthesis which results in the accumulation of squalene. This, in turn, would increase the level of farnesyl pyrophosphate and force the reaction towards the synthesis of ubiquinone.

This paper deals only with the biosynthesis of isoprenoid compounds from mevalonic acid. A second metabolic block due to vitamin A deficiency which results from the accompanying inanition occurs between acetate and mevalonate. This will be the subject of a later communication.

Acknowledgments

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CONTINUOUS METABOLIC STUDIES ON NORMAL AND DEPANCREATIZED DOGS DURING REPEATED WHOLE BODY X IRRADIATION[1]

O. H. Gaebler, R. J. Bloor, and Harold C. Choitz

Abstract

Experiments on normal and depancreatized bitches were carried out to determine how well multiple doses of 90 r or less of X radiation are tolerated, and whether there are any metabolic effects suggesting stimulation or suppression of the adrenals. In two normal animals, total dosage for the main portion of the trunk was 574 and 3549 r, in 4 and 22 months, respectively; in the depancreatized one, 807 r in 9 months. Dosages for head and pelvis approximated 44% of these amounts. Food intake was constant, and daily records were kept of body weight, water intake, urine volume, and urine nitrogen. The general condition of all animals remained excellent. Periods of estrus continued. Weight was maintained or increased during long series of exposures. No changes in water balance or nitrogen output occurred which resembled those observed in dogs receiving corticotropin or hydrocortisone (1). In the depancreatized animal, the insulin requirement, known for 5 preceding years, was unaffected. Thus no evidence for stimulation or suppression of adrenal function was obtained. Histological examination of bone marrow and other tissues of the animal which received the largest total dose gave little evidence of damage. Moderate increases in urine volume of the normal animals suggested possible early renal impairment.

Introduction

In a review published 10 years after the general adaptation syndrome was described, Selye (2) cited eight references which indicated that X rays and other ionizing radiation should be included among the diverse agents that elicit this response to injury. Since then, many studies have appeared which deal with: absence of the alarm reaction and diminished tolerance of radiation, in hypophysectomized or adrenalectomized animals; stimulation and exhaustion of the adrenals in intact subjects; and the validity of using corticotropin or adrenal hormones as palliatives. Some of the contributions prior to 1955 were discussed by French, Migeon, Samuels, and Bowers (3).

It seemed of interest to determine whether any evidence for adrenal stimulation or suppression is obtained with doses of X radiation which do not cause acute injury. We also wished to find out how often sublethal doses of considerable size can safely be repeated. Our studies in dogs deal with multiple doses of 45 to 90 r for the main portion of the trunk. Results obtained by others, with single doses of 100 to 300 r will be discussed in a later section. $LD_{50/30days}$ for dogs is usually stated to be about 350 r.

Our experimental approach was based on observations that corticotropin and hydrocortisone cause profound changes in weight, water balance, and nitrogen output in intact dogs (1), and that the insulin requirement of depancreatized

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Contribution from the Edsel B. Ford Institute for Medical Research, and the Department of Radiology, Henry Ford Hospital, Detroit, Michigan. This work was supported in part by research grant AT(11-1)-598 from the United States Atomic Energy Commission.

animals is greatly altered by either hypophysectomy or adrenalectomy (4, 5). Daily measurements which this metabolic approach involves should, of course, detect any derangement of homeostasis resulting from damage to tissues or organs other than the pituitary or adrenals.

Experimental

Adult bitches were used as experimental subjects. Details concerning composition of the diet and insulin therapy are given in a preceding study (6). Food intake, in grams, was uniform throughout the entire period of observations on each subject; values for nitrogen intake given in the tables vary slightly, being based on actual analyses of each lot of stock diet. Dietary protein consisted primarily of casein, with cracker meal as a minor source. Normal dogs 76 and 78 were trained for several months preceding the present experiments. The depancreatized animal, dog 63, had been used in numerous experiments (6, 7) during 5 postoperative years.

For exposure to X radiation, each dog was placed in a wooden box having the following dimensions, in inches: $34\frac{1}{2}$ long, $15\frac{1}{2}$ wide, $27\frac{1}{4}$ deep. A sling passing under the belly kept the animal at a uniform distance from the source. The box was on casters, and was wheeled into position under the source, so that radiation was applied from above, with the dog in a standing position. In nearly all instances, the interval between exposures was 10 days. Radiation was obtained from either of two X-ray generators operating at 200 kilovolts, with half-value layer of 1 mm of copper. Field intensity was measured in air, at a distance equal to that from the tube to the middle of the animal's body, and also by sacrificing the animal that had been used, or one of nearly the same size and weight, suspending it in the box, and placing the Victoreen meter successively in the mouth, pleural and peritoneal cavities, and vagina. Measurements in air served as a basis for calculating exposure time for intended dosages. Actual dosages recorded in the tables and mentioned in the text are those calculated for the main portion of the trunk, from measurement of field intensity in pleural and peritoneal cavities of sacrificed animals, and the known exposure time. For the head and pelvic regions, the values were about 44% as large.

Results in Normal Dogs

Dog 76

Soon after the first exposure of this animal to X radiation, the experiment was interrupted due to occurrence of estrus. During the subsequent 99-day period (Table I), eight exposures to doses of 60 to 65 r produced no deleterious effects. The animal remained vigorous and healthy. Gain in weight continued. Nitrogen was probably stored, since the average daily urine nitrogen was 2.0 g below intake, and fecal nitrogen rarely exceeded 1.3 g in numerous complete balance experiments in which the same diet was used. Little significance was attached to the increase in urine volume noted in this experiment, which was performed during warm months when some animals increase their water intake considerably. Daily records are presented in Fig. 1, for a period including the

second, third, fourth, and fifth total body X irradiation. There were no changes in weight, water balance, or urine nitrogen of the type that we observed after corticotropin or hydrocortisone (1).

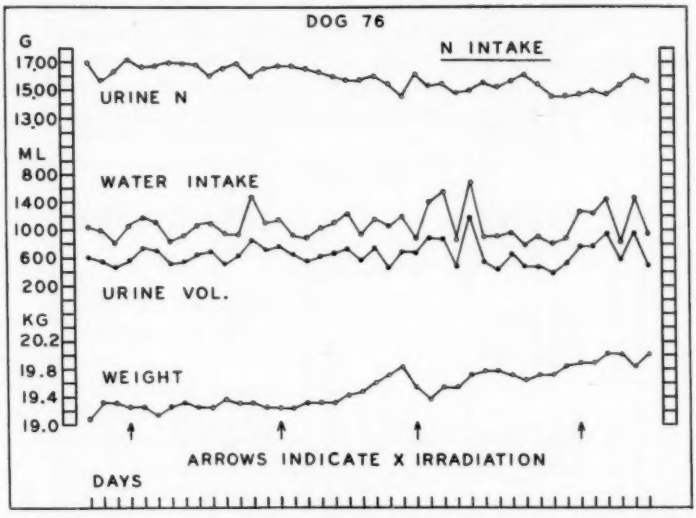

FIG. 1. Nitrogen output, water intake, urine volume, and weight of normal dog 76, during four exposures to 60–65 r of X radiation.

Dog 78

During 22 months, this large and rather sedentary animal was under daily observation for 530 days, in six periods separated by intervals of 10 to 43 days, and received a total dose of 3549 r, in 47 exposures (Table II). Our impression that gain in weight was due to slow accumulation of subcutaneous fat was in keeping with nitrogen data. During the first period of 120 days, average daily urine nitrogen was 1.84 g below intake, but in later periods this difference varied from 1.27 g to 1.58 g, and barely exceeded fecal nitrogen values obtained in complete balance experiments. The animal vomited 8 times during the third period of 210 days, and 3 times during subsequent intervals or periods. Vomiting never occurred on the day of exposure, or the following one, but happened at random from the 2nd to the 10th day, or even during long intervals between series of exposures. The animal remained very vigorous and healthy, and a relationship between rare instances of vomiting and X radiation seemed questionable. Estrus occurred between periods 2 and 3, 3 and 4, 5 and 6, of Table II.

An observed increase in average urine volume cannot, as in the case of dog 76, be considered a possible seasonal variation. It was not, however, as uniformly progressive as averages in Table II indicate. When the 210-day period was divided into 10 periods of 21 days each, average daily urine volume reached a

TABLE I

Data on weight, water balance, and urine nitrogen in normal dog No. 76

Periods of observation			Weight (kg)		Nitrogen intake (g/day)	Urine nitrogen (g/day)	Water intake (ml/day)	Urine volume (ml/day)	Exposure to X irradiation (r)
Starting date	Final date	Duration (days)	Initial	Final					
3-3-59	3-11-59	9	18.35	18.85	17.64	14.58	878	470	1×60–65
4-5-59	7-12-59	99	19.14	20.92	17.41	15.41	1208	699	8×60–65

TABLE II

Protocol and results of experiments on normal dog No. 78

Periods of observation			Weight (kg)		Nitrogen intake (g/day)	Urine nitrogen (g/day)	Water intake (ml/day)	Urine* volume (ml/day)	Exposure to X irradiation (r)
Starting date	Final date	Duration (days)	Initial	Final					
10-26-58	2-22-59	120	24.21	25.63	14.47	12.64	526	370	10×40–45
3- 5-59	3-29-59	25	25.68	26.08	14.49	13.01	530	319	1×60–65, 1×80–90
5- 4-59	11-29-59	210	26.37	28.63	14.39	12.87	637	446*	19×80–90
1- 1-60	2- 4-60	35	28.41	28.23	14.68	13.25	678	458	3×80–90
2-26-60	6- 9-60	105	28.80	29.43	14.63	13.36	777	513	10×80–90
7-23-60	8-26-60	35	28.63	29.14	14.69	13.11	717	529	3×80–90

*When period 3 was broken up into ten 21-day periods: 631, 459, 542, 484, 444, 381, 385, 369, 365, 397. Thus volume of urine was maximal at start of period 3, came all the way back to initial value, then rose.

maximum of 631 ml during the first of these 21-day periods, fell to 365 ml during the ninth one, and rose again thereafter.

Daily observations are presented in Fig. 2, for a 38-day period having the same midpoint as the 210-day period in Table II. Body weight had become nearly constant, and small decreases occurred after some exposures to X radiation. Following the second exposure, a day of very low nitrogen output was followed by unusually large water intake, urine volume, and urine nitrogen. Otherwise, day-to-day findings were essentially negative.

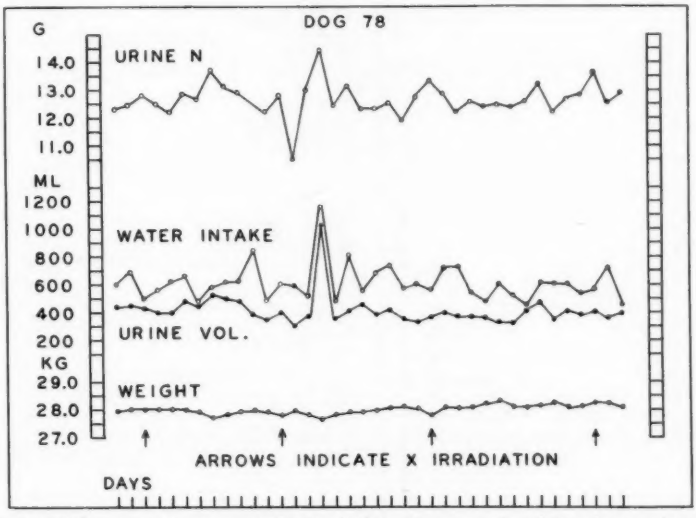

FIG. 2. Nitrogen output, water intake, urine volume, and weight of normal dog 78, during four exposures to 80–90 r of X radiation.

An autopsy of this animal was done by Dr. J. C. Hathaway, Jr., resident in Pathology in the Department of Laboratories, Henry Ford Hospital. There were no gross anatomical changes. Microscopic examination of bone marrow and other tissues also produced no evidence of extensive damage. Detailed description of the findings will be reported elsewhere.

Results in a Depancreatized Dog

Dog 63

During the year preceding experiments recorded in Table III, this animal excreted considerable glucose when insulin dosage was 28 units per day, and little or none with 30 to 32 units. The amount of insulin was intentionally kept at the lower level during the first period of 124 days (Table III), so that either an increase or a decrease in insulin requirement could readily be detected. Weight decreased, and glucose output was quite irregular (Fig. 3), as is usually

TABLE III

Results of experiments on depancreatized dog No. 63

Periods of observation			Weight (kg)		Nitrogen intake (g/day)	Urine nitrogen (g/day)	Water intake (ml/day)	Urine volume (ml/day)	Insulin dosage (units/day)	Urine sugar (g/day)	Exposure to X irradiation (r)
Starting date	Final date	Duration (days)	Initial	Final							
10-31-58	3- 3-59	124	18.97	16.41	14.35	12.00	1753	1315	28	18.2	11×40–45
4-10-59	7-11-59	93	16.53	17.38	14.41	11.97	1387	993	30	4.2	8×40–45

the case when insulin dosage is inadequate. No immediate relationship between glucosuria and exposures to X radiation was apparent. Total plasma lipid (8) and output of acetoacetic and β-hydroxybutyric acids (9) were determined for 3 days preceding and 2 days following the 11th exposure to X radiation at the end of the first period in Table III. Average postabsorptive values

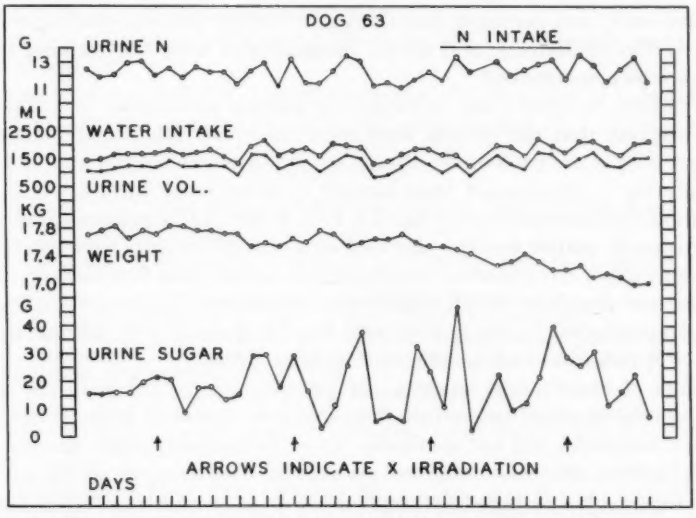

FIG. 3. Nitrogen output, water intake, urine volume, weight, and glucose output of depancreatized dog 63, during four exposures to 40–45 r of X radiation.

for total plasma lipids were 842 and 855 mg%; average daily output of acetone plus acetoacetic acid, 9.1 and 7.1 mg; and average daily output of β-hydroxybutyric acid, 15.3 and 12.6 mg. Thus the values preceding the final exposure in this period had not been elevated above the level expected in a slightly inadequately treated depancreatized dog, and the final exposure had no immediate effect. During the second period of 93 days (Table III), an increase of 2 units in the daily insulin dosage virtually abolished glucosuria, and gain in weight occurred during the series of exposures to X radiation.

Discussion

The total amounts of radiation which our dogs tolerated with apparent safety may seem large. In experiments on monkeys which received 100 r at 36-day intervals, Moon *et al.* (10) found that none survived more than 1200 r, or about 50% more than the single lethal dose for this species. That our dogs remained in excellent condition, after total doses that were large in comparison with the single lethal one of 350 to 400 r, may be due to such factors as lack of uniformity of the field, size of the animals, and size of the individual dose. In experiments of French *et al.* (3) on monkeys, field strength fell off only

5% at the extreme edges, and time of survival after single lethal exposures correlated with weight of the animal. In our experiments, the dose for head and pelvis was only 44% of that for the trunk, and the dog which received the largest total amount weighed up to 29 kilograms (Table II). In studies which were part of the University of Rochester Project (11), four dogs in a group of 20 survived 500 daily doses of 10 r, administered 6 days per week; leukopenia appeared early and persisted, but changes in serum protein, fibrinogen, blood NPN, serum cholesterol, and serum phosphatases were not striking even in animals which succumbed.

Our failure to obtain any evidence for adrenal stimulation or suppression might suggest that the criteria used were not valid. But water balance and nitrogen output are profoundly altered by corticotropin and adrenal hormones (1), differing in this respect from neutral 17-ketosteroid output, which is not altered by corticotropin in the dog (12, 13). Beher (12) also found that neutral 17-ketosteroid output was not elevated either in intact dogs which were given sufficient carbon tetrachloride to temporarily impair liver function, or in a depancreatized dog from which insulin was withdrawn. Absence of evidence for adrenal stimulation in the present experiments may simply indicate that the doses of X radiation were insufficient to cause stress.

Results of experiments on dogs, carried out in other laboratories, suggest that the dose required to produce demonstrable effects is large. Single 100-r or 200-r exposures did not markedly lower hemoglobin, although the latter amount greatly delayed hemoglobin restoration after hemorrhage (Brooks *et al.* (14)). Craddock *et al.* found that replenishment of leucocytes (15) and platelets (16), after depletion, was impaired by 250 r, but stated that this amount was delivered from two heads and corresponded to LD_{50}, or 350 to 400 r administered from one source. Lawrence (17) lost one dog in a series of 11 which received 300 r, indicating that this was a minimal lethal dose. The metabolic response which he obtained was apparently not an increase in neutral 17-ketosteroid output, as he supposed (18). Jackson and Entenman (19), using a procedure in which 250 r was near LD_{50}, observed a marked increase in adenine excretion after single doses of 500 r, but not after 200 r; a rise in uric acid plus allantoin occurred after 150 r.

Our experimental procedure was not designed for detection of long-term effects, such as slowing of growth in young animals, shortening of life span in adult ones, and development of renal or other lesions (20). If the increasing urine volumes observed in several animals (Tables I and II) were the result of early renal impairment, the damage caused by whole body irradiation must differ from that caused by strong beams directed toward the kidneys. In the well-known experiments of Hartman, Bolliger, and Doub (21), urine volumes remained unchanged during an initial acute nephritis, after which marked polyuria appeared.

Since determination of the minimal dose of X radiation that disturbs water balance or nitrogen metabolism is of some interest, we hope to carry out further

experiments in which the field is more uniform, and the beam directed horizontally, with the animal standing, so that the kidneys are not in a stronger field than other organs.

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FORMATION OF LABELED SUGARS FROM L-TYROSINE-C^{14} IN SOME HIGHER PLANTS[1]

R. K. Ibrahim, Sara G. Lawson, and G. H. N. Towers

Abstract

Radioactive sugars and organic acids, including glutamic and aspartic acids, were synthesized in leaf disks of five species of angiosperms from administered uniformly labeled L-tyrosine. The synthesis of sugars was appreciable in light; in the dark there was virtually no conversion to carbohydrate. In three different experiments, 28%, 36%, and 46% of the total activity of tyrosine-U-C^{14}, administered to leaf disks of *Pyrus communis*, could be accounted for in identifiable aliphatic compounds. These results indicate that tyrosine is a glucogenic amino acid in higher plants and suggest also that three or more carbons of the tyrosine molecule are utilized. Ring cleavage is a possibility.

Introduction

In an investigation of phloridzin synthesis in *Malus*, Hutchinson *et al.* (1) reported that sugars were synthesized from administered tyrosine. Dougall and Shimbayashi (2), in studies of tyrosine metabolism in tobacco callus tissues, found that 3.6% of the administered tyrosine-C^{14} was converted to $C^{14}O_2$ in a 24-hour period. Only a fraction of the cell wall residue could be accounted for, after hydrolysis, by tyrosine residues. They assumed that a large portion of administered tyrosine had been utilized to form lignin. Brown and Neish (3), on the other hand, have reported that the ability to synthesize lignin from tyrosine in higher plants appears to be restricted to the Gramineae.

In the course of a study of arbutin biosynthesis in *Pyrus communis*, which is being carried out in this laboratory (4), our attention was drawn to the prominence of labeled sugars on chromatograms of ethanol extracts of leaf disks which had been administered tyrosine-U-C^{14}. We considered it of interest to determine the extent to which the carbon of tyrosine is utilized in the synthesis of non-aromatic compounds. An attempt was made, therefore, to draw up a balance sheet of the radioactivity incorporated into various non-aromatic fractions of *Pyrus communis* leaf disks which had been administered tyrosine-U-C^{14}.

Materials and Methods

Leaf disks were obtained from the following species of plants grown in the greenhouse: *Pyrus communis* L., *Pelargonium hortorum* L. H. Bailey, *Grevillea robusta* A. Cunn., *Gaultheria procumbens* L., and *Triticum vulgare* var. *Thatcher*.

Uniformly labeled L-tyrosine-C^{14} was purchased from Merck & Co., Montreal.

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Contribution from the Botany Department, McGill University, Montreal, Que.

1. Administration of Tyrosine-C^{14} and Extraction of Plant Material

Leaf disks were obtained with a Ganong leaf punch from *Pyrus*, *Gaultheria*, and *Pelargonium*. With *Grevillea* and *Triticum*, leaf sections were used.

Leaf material was vacuum infiltrated with tap water and floated on aqueous solutions of tyrosine-C^{14} in a covered Petri dish in the dark or under a bank of fluorescent lights for various periods. At the end of these periods they were washed twice with tap water and rapidly killed in boiling 80% ethanol. The residual tyrosine solution and the washings were combined, made to volume, and their radioactivity determined. The leaf material was extracted under reflux, with three changes of ethanol. The combined ethanol extracts were evaporated to dryness under a jet of filtered air and the residue was redissolved in ethanol to a measured volume. Aliquots of the ethanol extract were chromatographed and assayed for radioactivity. Leaf disks of *Pyrus communis* were further extracted under reflux with benzene and the activity of the benzene extract was determined. Following extraction with benzene, these disks were dried in a vacuum desiccator, weighed, and ground to a fine powder. Radioactivity determinations were made on 10- to 20-mg samples of this powder.

2. Hydrolysis of the Insoluble Residue of Pyrus communis Leaf Disks

(*a*) Samples consisting of 80–100 mg of the powdered residue were treated with 0.8–1.0 ml of 72% H_2SO_4 (w/w) for 2 hours with stirring. The mixture was transferred to a flask with 84 ml water, boiled gently for 12 hours under reflux (5), and filtered through a small disk of Whatman No. 1 paper on a sintered glass funnel. The disk was dried and combusted for radioactivity. This portion was labelled "lignin fraction". The filtrate and washings were passed successively through a bed of IR-45(OH^-) and IR-120(H^+). The resulting deionized solution was taken to dryness on a flash evaporator, and the residue was made to volume with 50% ethanol. Aliquots of this "insoluble carbohydrate fraction" were chromatographed and assayed for radioactivity.

(*b*) Samples consisting of 80–100 mg of the powdered residue were refluxed with 250 ml 6 *N* HCl for 24 hours (6). The hydrolyzate was filtered and the filtrate taken to dryness under an air jet. The residue was dissolved in water and passed through a bed of IR-120 (H^+) from which amino acids were eluted with 4% NH_4OH. This eluate constituted the "protein amino acid fraction".

Tyrosine was found to be stable to this procedure. This was tested by hydrolyzing 88 mg of inactive pear leaf insoluble residue to which 1.18 μc of standard tyrosine-U-C^{14} had been added. The recovery of tyrosine as determined by radioactivity measurements was $89 \pm 1\%$.

3. Chromatography and Radioactivity Determination

Total ethanol extracts, carbohydrate fractions, and protein amino acid fractions were chromatographed two-directionally on large sheets of Whatman No. 1 chromatography grade paper. The solvent for the first direction was (4:1) phenol:water, and for the second, the upper phase of a mixture of (4:1:5)

n-butanol:acetic acid:water. Air-dried chromatograms were radioautographed for 1–2 weeks using Kodak no-screen X-ray film.

Radioactive spots on chromatograms were counted directly on the paper by means of a Nucleonics type IWAAA thin end-window G.M. tube attached to a Berkeley Decimal Scaler. The identity of radioactive sugars, amino acids, and organic acids was confirmed by spraying the chromatograms with reagents specific to these compounds. Aliquots of soluble and insoluble fractions were oxidized to $C^{14}O_2$ with Van Slyke reagent and measurements of radioactivity were made with a Nuclear Chicago model 600 dynamic condenser electrometer (Dynacon) incorporating an ion chamber. Samples were combusted in duplicate with an error of less than 4%.

Experiments and Results

Three separate experiments were carried out in which tyrosine-U-C^{14} was administered.

Experiment 1.—Sets of leaf disks of *Pyrus*, *Pelargonium*, and *Gaultheria* and leaf sections of *Triticum* and *Grevillea* were each administered 2 μc of tyrosine-U-C^{14} for various periods in the light. Other sets of leaf material of *Pyrus*, *Pelargonium*, and *Triticum* were fed the same amount of labeled tyrosine in the dark. Chromatograms of the 80% ethanol extracts were radioautographed and the activity was determined by direct counting. The results are presented

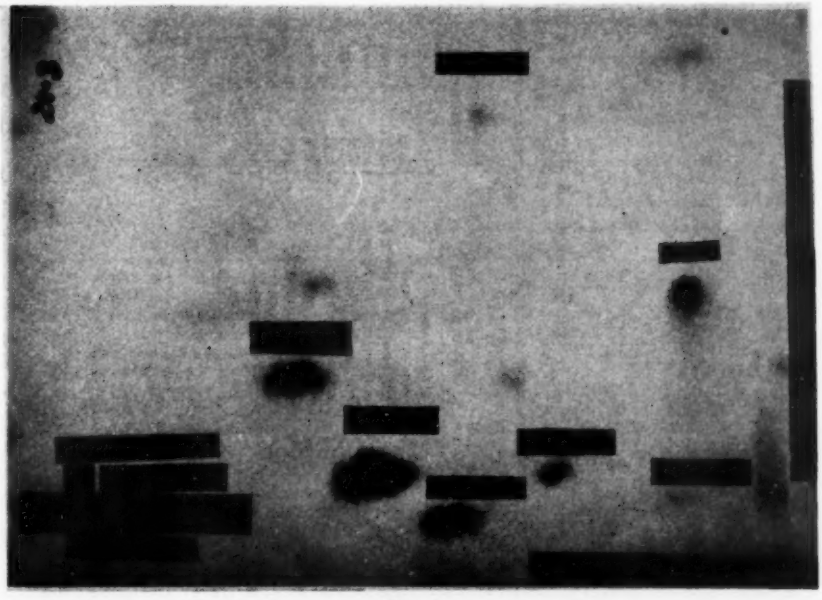

FIG. 1

TABLE I

Distribution of radioactivity in 80% ethanol soluble fractions of leaf material of different plants administered tyrosine-U-C^{14}

Expt.	Plant species	Conditions of experiment	Percentage distribution of radioactivity				
			Sugars	Glutamic	Aspartic	Malic	Tyrosine
1	*Pyrus communis*	Light, 17 hours	67	3	1	7	13
1	*Pyrus communis*	Dark, 17 hours	0	4	1	3	71
1	*Pelargonium hortorum*	Light, 24 hours	40	0	Trace	24	3
1	*Pelargonium hortorum*	Dark, 24 hours	4	1	0	0	82
1	*Triticum vulgare*	Light, 24 hours	34	6	1	6	12
1	*Triticum vulgare*	Dark, 24 hours	0	1	0	0	89
1	*Grevillea robusta*	Light, 19 hours	20	2	0	29	23
1	*Gaultheria procumbens*	Light, 24 hours	40	0	0	30	2
2	*Pyrus communis*	Light, 17 hours	43	2	0	15	7
3	*Pyrus communis*	Light, 6 hours	20	2	1	28	31
3	*Pyrus communis*	Light, 23 hours	30	2	Trace	16	3

in Table I. A radioautograph of the chromatographed 80% ethanol extract of *Pyrus* administered tyrosine-C^{14} in light is shown in Fig. 1.

Experiment 2.—A set of 88 leaf disks was administered 0.157 mg tyrosine-U-C^{14} (5.27 μc) in 3.4 ml water for 17 hours in the light. The distribution of activity in the various fractions is given in Table II. The distribution of activity in some of the compounds identified in the 80% ethanol soluble fraction is given in Table I. Table III shows the percentage conversion of tyrosine-C^{14} to non-aromatic compounds.

TABLE II

Distribution of radioactivity in various fractions of *Pyrus communis* leaf disks administered tyrosine-U-C^{14} in light

Distribution of activity	Expt. 2, 17 hours (μc)	Expt. 3	
		6 hours (μc)	23 hours (μc)
(*a*) Administered	5.27	5.93	5.93
(*b*) Remaining in medium	0.21	2.46	0.37
(*c*) Absorbed by disks ($a-b$)	5.06	3.47	5.56
(*d*) Ethanol-soluble fraction	1.83	1.74	2.39
(*e*) Benzene-soluble fraction	0.003	0.003	0.000
(*f*) Insoluble residue	3.07	1.57	2.21
(*g*) Unaccounted for ($c-(d+e+f)$)	0.16	0.16	0.96
(*h*) Lignin	—	0.17	0.22
(*i*) Insoluble carbohydrates	0.21	0.05	0.08
(*j*) Protein amino acids	1.59	0.14	0.15
(*k*) Unaccounted for in insoluble residue ($f-(h+i+j)$)	1.27	1.21	1.76

TABLE III

Conversion of tyrosine-U-C^{14} to non-aromatic compounds in *Pyrus communis* leaf disks in light

Distribution of activity	Expt. 2, 17 hours (μc)	Expt. 3	
		6 hours (μc)	23 hours (μc)
(*a*) Total of ethanol-soluble and ethanol-insoluble activity	4.90	3.31	4.60
(*b*) Tyrosine in ethanol-soluble fraction	0.11	0.53	0.07
(*c*) Tyrosine in ethanol-insoluble fraction	1.32	0.11	0.12
(*d*) Metabolized tyrosine ($a-(b+c)$)	3.47	2.67	4.41
(*e*) Identified non-aromatic compounds:			
Sucrose and glucitol	0.78	0.34	0.72
Carbohydrate fraction	0.21	0.05	0.08
Free amino acids	0.04	0.06	0.03
Protein amino acids	0.27	0.02	0.03
Malic acid	0.28	0.49	0.37
Total	1.58	0.96	1.23
(*f*) Conversion of tyrosine to non-aromatic compounds ($(e/d)\times100\%$)	45.6%	36.0%	28.2%

Experiment 3.—Two sets of disks were each administered 0.173 mg (5.93 μc) tyrosine-U-C^{14} in 3 ml of water. One set (88 disks) was fed for 6 hours the

other (103 disks) for 23 hours, both in light. The distribution of radioactivity in various fractions is given in Table II and the percentage distribution of activity in the ethanol-soluble fraction is given in Table I. The percentage conversion of tyrosine-C^{14} to non-aromatic compounds is shown in Table III.

Discussion

Table I shows that sugars were formed readily in leaf material from tyrosine administered in light, whereas there was almost no synthesis in the dark. In this respect, the fate of tyrosine resembles that of succinate (7), and acetate (8) in green tissues, and suggests that tyrosine gives rise to acetate. The presence of malic, aspartic, and glutamic acids further supports this view. A mechanism for the conversion of acetate to sugars has been suggested by Beevers (8).

There are four possible ways in which organic acids and sugars may be derived from tyrosine: (1) from a one-carbon unit (arising by decarboxylation of tyrosine followed by refixation in photosynthesis), (2) from a two-carbon fragment equivalent to acetate, (3) from the three-carbon side chain, or (4) by ring cleavage and utilization of all the carbons of the tyrosine molecule. The quantitative data presented in Table III indicate that 46%, 36%, and 28% of the activity of the tyrosine-C^{14} was incorporated into aliphatic compounds. If the tyrosine used in these experiments was genuinely uniformly labeled, then removal of two carbons would result in a maximum of 22% of the total activity being distributed in non-benzenoid compounds. Since the three values for the percentage of conversion of tyrosine to aliphatic compounds are all higher than this, the first two possible mechanisms can be eliminated. Also, there was no evidence of C_6–C_1 or C_6–C_2 phenolic compounds with any activity on chromatograms (see Fig. 1), which supports the view that more than two carbons of tyrosine must be utilized.

If the three-carbon side chain of tyrosine were utilized, 33.3% of the tyrosine activity should occur in non-aromatic compounds. This is in the range of activities actually found. However, there was no evidence of activity in phenols. Pear leaves contain rather large amounts of arbutin, the β-glucoside of hydroquinone, but this compound never had more than a trace of activity in it.

Ring cleavage of tyrosine-U-C^{14} would be indicated if more than 33.3% of the activity were found in non-aromatic compounds. Table III shows that this is a possibility in two of the three experiments. Ring cleavage of tyrosine has been demonstrated in microorganisms (9) and in rats (10, 11). One of the products is malic acid and the other is acetoacetate, which is readily converted to acetate. Table II shows that malic acid was a prominent radioactive compound in the ethanol-soluble fraction. The synthesis of aspartic and glutamic acids from acetate is a characteristic feature of acetate metabolism, and Table II shows that these two amino acids were labeled in most of the experiments. There is thus some reason for believing that a pathway for the cleavage

of the ring of tyrosine may exist in higher plants, similar to that which has been demonstrated in rats. This question could be resolved more satisfactorily by the administration of ring-labeled tyrosine, but this compound is not available commercially at the present time.

Table II (line *k*) shows that 20–30% of the total activity absorbed by the leaf disks of *Pyrus communis* could not be accounted for by amino acids, sugars, or 'lignin'. This activity resided in the ethanol- and benzene-insoluble residue. The radioactive compound or compounds which accounted for this activity were solubilized by the treatment of residues with 72% H_2SO_4. Neither acid hydrolysis (refluxing with 2 *N* HCl for 3 hours) nor alkaline hydrolysis (treatment with cold 2 *N* KOH for 24 hours) of the residues produced phenolic acids with appreciable radioactivity. The nature of this ethanol-insoluble and highly radioactive material synthesized from tyrosine was not studied further. The reviewer of this paper has suggested that this material might be melanin, formed by the action of tyrosinase.

It is clear from Table I that 10 times as much tyrosine was found in the acid hydrolyzate of the insoluble residue of the 17-hour feeding (experiment 2) as from the same fractions in the 6-hour and 23-hour feedings (experiment 3). This may reflect the age of the leaves used in these experiments. The plant material for experiment 3 consisted of tough, mature leaves whereas considerably younger leaves were used in experiment 2. Other differences are also apparent in this table.

Although the activities of C^{14} which could not be accounted for amounted to only about 5% of the total activities administered in experiment 2 and the 6-hour feeding of experiment 3 (see Table II), the loss is more serious in the 23-hour feeding of experiment 3. Since the disks were administered tyrosine-C^{14} in the light, it is difficult to see how this loss could be due to $C^{14}O_2$ emission. This may be the result of the production of volatile compounds by the leaf, which were lost on fractionation or on chromatography of the extracts.

Our results show that in some higher plants the fate of tyrosine and phenylalanine is quite distinct. Tyrosine is readily utilized in the synthesis of sugars in light, whereas phenylalanine, administered to *Pyrus*, is converted to cinnamic acid derivatives such as chlorogenic acid (4) with only a very minor conversion to organic acids, even in the light. There is virtually no formation of cinnamyl compounds in *Pyrus* from tyrosine. We have been unable to detect any evidence of the interconvertibility of tyrosine and phenylalanine in leaf disks of *Pyrus communis*.

The possibility of conversion of tyrosine to cinnamyl compounds in the other species studied here was not investigated. In wheat, fast-moving radioactive compounds were detected on chromatograms of ethanol extracts after administration of tyrosine-C^{14}. The positions of these spots on chromatograms suggest that they could be derivatives of cinnamic acids. In this connection, it has been shown by Neish (12) that wheat contains an enzyme, tyrase, which converts L-tyrosine to *p*-coumaric acid.

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THE RESOLUTION OF URINARY OR SERUM PROTEINS BY CHROMATOGRAPHY ON DEAE CELLULOSE COLUMNS WITH PARTICULAR REFERENCE TO URINARY PROTEINS AFTER THERMAL BURNS[1]

S. H. JACKSON, A. W. FARMER, R. J. SLATER, AND M. S. DEWOLFE

Abstract

Serum proteins were separated into the usual electrophoretic fractions by the starch block technique. Each fraction was then examined by DEAE cellulose chromatography and by starch gel electrophoresis. The combination of starch block electrophoresis and DEAE cellulose chromatography was able to distinguish 22 different serum proteins.

The application of DEAE cellulose to the chromatographic resolution of urinary proteins is described. The urine was filtered and the protein was concentrated by ultrafiltration. After dialysis, a suitable aliquot was adsorbed on a DEAE cellulose column and eluted by buffers with a progressively increasing hydrogen ion concentration and ionic strength. Some illustrative chromatographs are given.

A number of urines from patients with severe burns were examined by this procedure. The increase in protein excretion was found in all sections of the chromatographs with particular increases in the γ globulin and acid mucoprotein zones.

Introduction

The examination of the proteins occurring in urine has been largely dependent on separation into electrophoretic fractions related to the classical fractions of serum proteins, sometimes supplemented by further identification with serum proteins by immunochemical means (1, 2, 3, 4, 5, 6). Usually moving boundary electrophoresis at pH 8.6 was used (1, 2, 3, 4, 5), although Boyce, Garvey, and Norfleet (1), and Webb, Rose, and Sehon (5) also studied the migration of the acidic mucoproteins at pH 4.5. In the normal urinary protein electrophoretic pattern, while the albumin peak was well delineated, the globulin fractions were not nearly so clearly separated as in the usual serum pattern. Indeed, they were divided into the globulin components more by reference to the serum pattern than by distinguishing features of the urinary protein pattern.

Grant (2) combined electrophoresis in agar gel with immunological detection and was able to demonstrate the presence in normal urine of components that were electrophoretically and immunologically similar to most of the serum electrophoretic fractions. However, he did observe duplication of urine components antigenically related to γ globulin.

Webb, Rose, and Sehon (6) separated the proteins of urine by electrophoresis on starch blocks. The components in the albumin, and α_1, α_2, β, and γ_2 zones,

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were recovered and examined by immunochemical means and by ultracentrifugal analysis. Although each had similar antigenic properties to the corresponding serum fraction, the average molecular weight of each urinary globulin fraction was less than the corresponding serum fraction. This was particularly true of the γ_2 globulin fraction, which had an estimated molecular weight of 10,600.

Early in our studies of urinary protein it became clear that some method of separation with greater resolution than the usual electrophoretic procedure was necessary. Sober, Gutter, Wyckoff, and Peterson (7) and Peterson and Sober (8) had published methods for the synthesis of substituted cellulose derivatives suitable for use in ionic exchange fractionation and had illustrated their application to the separation of serum proteins. In 1958 Sober and Peterson (9) reviewed some of the fractionations which had been achieved with their cellulose columns.

This report concerns the application of diethylaminoethyl cellulose (DEAE cellulose) to the fractionation of the proteins in normal urine and in some types of proteinuria.

In order to relate the various urinary proteins to serum proteins, a study of the further resolution of the electrophoretic fractions of serum by DEAE cellulose chromatography and by starch gel electrophoresis was undertaken.

Various authors (9, 10) have separated total serum proteins by DEAE cellulose chromatography and examined the fractions so obtained by paper electrophoresis. While some fractions from the DEAE cellulose column were found to be electrophoretically pure, other fractions proved to be mixtures of several components. Indeed, the major albumin peak contains, in addition to the albumin, α_1, α_2, and β components. It was therefore reasoned that prior separation of the total serum proteins by electrophoresis on a starch block would lead to a clear resolution of the proteins by DEAE cellulose chromatography.

A further aid to the definition of the DEAE cellulose fractions would be obtained by the simultaneous separation of the starch block electrophoretic fractions by Smithies' starch gel electrophoretic technique (11).

An increased urinary nitrogen excretion after thermal burns has long been recognized (13). A large proportion of this nitrogen appears in the form of high molecular weight complexes (13, 14). An increase in the serum mucoproteins after thermal burns has been well demonstrated (15, 16, 17, 18).

It is probable that this results in an increased urinary excretion of these substances. Rosenthal *et al.* (19) have demonstrated that skin slices, when scalded in hot water, liberate gelatin and a protein complex containing hexose and sialic acid. Gjessing and Chanutin (20) found an increase in the α_2 globulins in scalded rats. There is a possibility that any of these proteins might also be found in the urine of burn patients. In view of these observations it seemed to be advisable to examine the urinary proteins, particularly the mucoproteins, in cases of severe burns.

Experimental

(a) Collection and Concentration of Urine

Urine was collected into a bottle containing 2 ml of 10% sodium azide solution and 1 g of thymol. Whenever possible, it was kept refrigerated during collection although this is not essential. The urine was stored in the refrigerator until processed. Freezing was found to alter the protein pattern.

Twenty-four-hour collections from burn patients were made by catheter for the first 10 days of treatment. The urine specimen was filtered through a thin layer (1 to 2 mm) of Hyflo-Supercel on a suction filter. In order to remove heavy metals into the ultrafiltrate in the next step, 1 g of EDTA was added per liter of filtrate. Care was taken not to shake the urine vigorously in order to minimize surface denaturation.

The proteins were concentrated by ultrafiltration using Visking cellulose tubing as proposed by Grant (2). About 12 feet of 8/32 in. tubing was used without tube-gauze support. A negative pressure of 350 mm of Hg was applied. This was controlled by a spring-loaded bypass valve in the suction line. A negative pressure of over 400 mm resulted in sufficient distention of the tubing that larger molecules could pass through and albumin could be detected in the ultrafiltrate. A pressure of less than 300 mm gave a very slow filtration rate. At 350 mm, a liter of urine could be concentrated in 24 hours.

The urine protein concentrate, which contained roughly 1% of protein, was dialyzed for 2 to 3 days, at refrigerator temperature, against repeated changes of 0.005 *M* phosphate buffer at pH 7.0.

After dialysis, the concentration of protein was determined by the method of Lowry *et al.* (21) using a standard of human serum albumin.

(b) Chromatographic Procedure

The anion exchanger, DEAE cellulose, was chosen as we were particularly interested in separating the acid mucoproteins. The procedure was essentially that described by Peterson and Sober (8).

DEAE cellulose (Eastman No. 7392), after regeneration with 0.5 *N* sodium hydroxide containing 0.5 *N* sodium chloride, was equilibrated against the starting buffer (0.005 *M* phosphate buffer at pH 7.0). The column was 400 mm long and 10 mm inside diameter. It was jacketed so that it could be cooled with water at 6–8° C. The DEAE cellulose, in a thin slurry in the phosphate buffer, was poured into the column and allowed to settle by gravity until the cellulose column was 300 mm long. Further DEAE cellulose was added and packed under 10 lb of air pressure until the packed column was again 300 mm long. The column capacity, when titrated between pH 7.0 and 4.0, was about 1.2 meq. However, it was found that different batches of DEAE cellulose had different protein adsorption capacities for equivalent anion exchange capacity. Hence conditions had to be adjusted for each batch of DEAE cellulose. The column was equilibrated overnight in the cold by pumping starting buffer through it at a rate of 5 ml per hour.

An amount of protein concentrate found to contain the equivalent of 5 to 25 mg of albumin by Lowry's procedure was placed on the DEAE cellulose column and allowed to adsorb by gravity. The eluting buffer was then pumped through the column by a Sigmamotor pump regulated to deliver a constant amount of 5 to 6 ml per hour. Fractions were collected in a fraction collector set to change tubes at such a time interval that exactly 5 ml was collected in each fraction. The entire procedure was carried out in a cold room. Since use of the room during the day tended to raise the temperature, cold water circulation was used to maintain the temperature of the column itself at about 6 to 8° C.

A variety of gradient systems for varying the concentration of the buffer was tried (22). Typical gradients are given in Figs. 3L and 5H.

The total protein in each fraction was determined by a macromodification of the method of Lowry *et al.* (21). The results, as optical density readings, were plotted against the total volume of eluate collected. This constituted the chromatograph in which the protein was expressed as "optical density units". These could be readily converted to "mg equivalent albumin", by reference to the calibration of the method against human albumin.

The fraction of the total protein in a single peak or area of the chromatogram is represented by the ratio of this area to the total area of the chromatogram. The recovery of total protein is calculated from the ratio of the total area to the total calculated area. The total calculated area is derived in the following manner. The vertical optical density scale of our graph paper was 0.2 unit per inch. The horizontal scale of volume of eluate was 50 ml per inch. Hence 1 square inch of graph area was 10 arbitrary optical density units. It was found by reference to the calibration of the Lowry protein determination that 25 mg of albumin, i.e. the total amount of protein absorbed on the column, was equivalent to 230 arbitrary optical density units, hence to 23 square inches. Therefore, a total chromatograph area of 23 square inches would represent 100% recovery.

The sialic acid of each fraction was determined by the diphenylamine reaction as applied by Anderson and Maclagan (23). A secondary standard of α_1 glycoprotein, prepared from plasma by the method of Weimer, Mehl, and Winzler (24), was used. The sialic acid composition of the secondary standard was determined by comparison with pure N acetyl neuraminic acid.*

Wherever indicated the fractions were also tested for hexuronic acids by the carbazole method of Dische (25). A glucuronic acid standard was used. In some cases humin formation rendered the test useless.

Various procedural tests were applied to check the reliability of this method.

The porosity of the ultrafiltration tubing was tested by filtration of a partially hydrolyzed solution of dextran. Alcohol was added to the ultrafiltrate slowly, with stirring, until the first traces of turbidity formed. The dextran that was precipitated at this point, i.e. the ultrafilterable dextran with the highest

*Kindly supplied by Dr. Gunnar Blix (University of Uppsala, Sweden).

molecular weight, was collected, redissolved in water, and lyophilized. The approximate molecular weight was determined both by the analytical ultracentrifuge and by the viscosity method of Wolff *et al.* (26). The ultracentrifugal method gave an average molecular weight of 20,000. The viscosity measurement gave a number average molecular weight of 14,460 and a mean weight molecular weight of 19,060, the latter agreeing well with the ultracentrifugal value.* One can therefore expect that, while the smaller peptides would be lost, anything over about 20,000 molecular weight will be retained by this procedure.

Possible formation of artifacts during ultrafiltration was tested by concentrating serum that had been diluted 2000-fold with normal saline. The chromatograph prepared from this serum agreed within experimental variation with the chromatograph prepared from the undiluted serum (Fig. 1).

Recovery of pure protein preparations from the chromatographic procedure averaged 95–99%. Albumin,† α_1 glycoprotein,‡ and ceruloplasmin§ were tested.

Recovery of urine protein concentrates ranged from 70–90%, averaging about 80%. The patterns obtained were reproducible in respect to the main peaks but fine detail of the shape of the peaks was variable.

Reconcentrating and rechromatographing the complete eluate of a urine protein concentrate did effect some changes in the chromatographic pattern that were beyond experimental variation (Fig. 2). There seemed to be a transfer of material to form a new peak at 430 ml. One might speculate that acidic components were split off the initial peaks. We had observed that dialysis of urine prior to ultrafiltration results in the gross production of artifacts. There seems to be some protective material in urine that is lost during dialysis. This protection would not be present during the second ultrafiltration process so that some alteration could be expected in this step. We, therefore, attribute the variation in pattern to the second ultrafiltration under conditions which do not obtain in the usual procedure.

The separation of serum proteins by starch block electrophoresis followed by DEAE cellulose chromatography was done in the following manner:

Twenty milliliters of serum from a fasting normal adult male was separated by the starch block technique of Kunkel and Slater (27). A block 25 cm wide and 1 cm thick was used and the proteins were run for a distance of 40 cm. A narrow longitudinal strip was removed near one edge and 1-cm sections were analyzed for protein by the biuret procedure. The electrophoretic pattern was plotted from these results.

The remainder of the starch block was divided transversely into nine sections.

*This determination was made by Dr. E. S. Goranson (Princess Margaret Hospital, Toronto, Ontario).

†Armour preparation of human albumin.

‡Prepared by K. Schmid (Harvard Medical School, Boston) and ourselves.

§Prepared by Dr. A. F. Charles (Connaught Laboratories, University of Toronto, Toronto, Ontario).

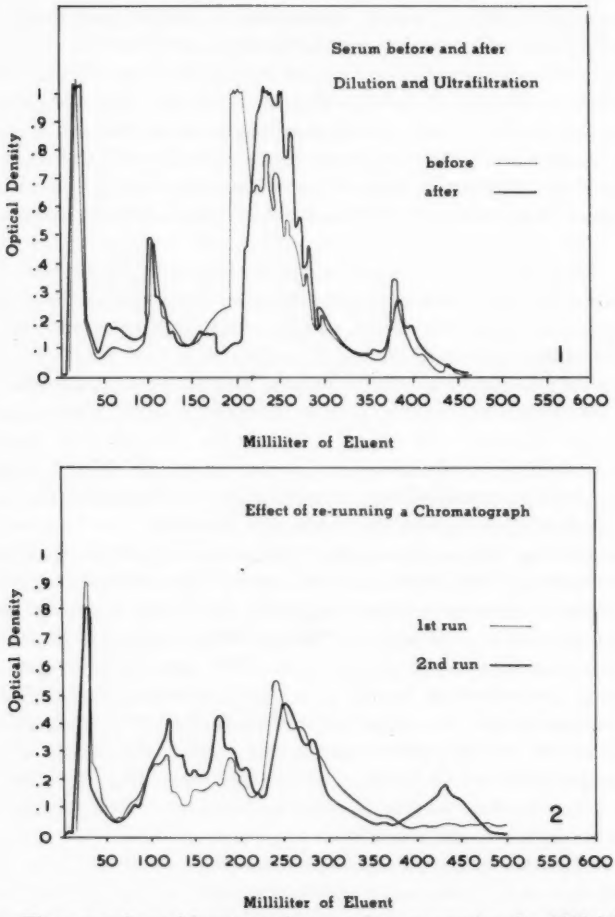

FIG. 1. The comparison with untreated serum of serum proteins after 2000-fold dilution in normal saline and concentration by ultrafiltration.
FIG. 2. The effect of reconcentrating and rechromatographing urinary proteins after initial chromatography on DEAE cellulose.

The divisions were made both on the basis of the electrophoretic pattern and on the basis of certain distinct colored bands. Each section was extracted with the veronal buffer used for the separation. Each extract was then concentrated by ultrafiltration and dialyzed against 0.005 *M* phosphate buffer.

An amount of extract found to contain 5 mg of protein by the Lowry procedure (21) was adsorbed on DEAE cellulose and fractionally eluted by the procedure previously described. The gradient elution of buffer concentration is illustrated in Fig. 3L. Fraction I (prealbumin) contained less than 5 mg of

protein so that the whole fraction was used.

The remainder of the starch block extracts was further concentrated by dialysis against polyvinyl pyrrolidone to about 3% protein. Aliquots of these concentrates were carried through the starch gel electrophoresis by the technique of Smithies (11).

Results

Serum Proteins

The DEAE cellulose fractionations of the starch block electrophoretic components are depicted in Fig. 3. The starch block electrophoretic pattern is shown in Fig. 3A. The nine fractions into which the pattern was divided for the DEAE cellulose fractionations are marked on this pattern. Figure 3B is the DEAE cellulose chromatogram obtained from the whole serum protein. Figures 3C to 3K inclusive are the DEAE cellulose chromatograms of individual electrophoretic fractions. The portion from which the chromatogram is obtained is indicated by the stippled figure accompanying each chromatogram.

In Fig. 4 the starch gel patterns are aligned with the portions of the starch block separation from which they are derived. A starch gel pattern of the whole serum is included for comparison.

Fraction I is the prealbumin fraction. DEAE cellulose separated this fraction into two components (Fig. 3C). The first of these has the main peak at 330 ml of eluate. The second component, with the peak at 425 ml, is the last, and hence probably the most strongly anionic component, to be found clearly defined in any serum fraction. This is borne out by the starch gel pattern. Note that in the pattern of the complete serum protein (Fig. 4) there are two small prealbumin components to be seen. One of these is proximal to the albumin, but the other has moved further toward the anode. The eluate from the DEAE cellulose was reconcentrated and run on the starch gel (Fig. 4I). It demonstrated that the most advanced prealbumin in the starch gel pattern of the whole serum proteins was a component of fraction I. It was suspected that this component might be an acid mucopolysaccharide, but tests for hexuronic acids and for "sialic" acid were both negative.

Fraction II is the first half of the albumin peak. The peak was divided at this point since this section of the starch block was a red color, while the second half of the peak was yellow. The material responsible for the red color has not been identified. DEAE cellulose chromatography revealed two components in this fraction, although they were not well separated (Fig. 3D). The starch gel pattern of this fraction demonstrated only a single component (Fig. 4, II).

Fraction III comprises the second half of the albumin peak. This section was yellow, probably due to bilirubin. The DEAE cellulose chromatograph showed a single peak with a slight shoulder on the trailing edge (Fig. 3E). The starch gel fractionation revealed three components (Fig. 4, III). The great bulk of material was albumin, but some of the prealbumin that was observed in the total serum protein pattern, proximal to the albumin, is found in this fraction

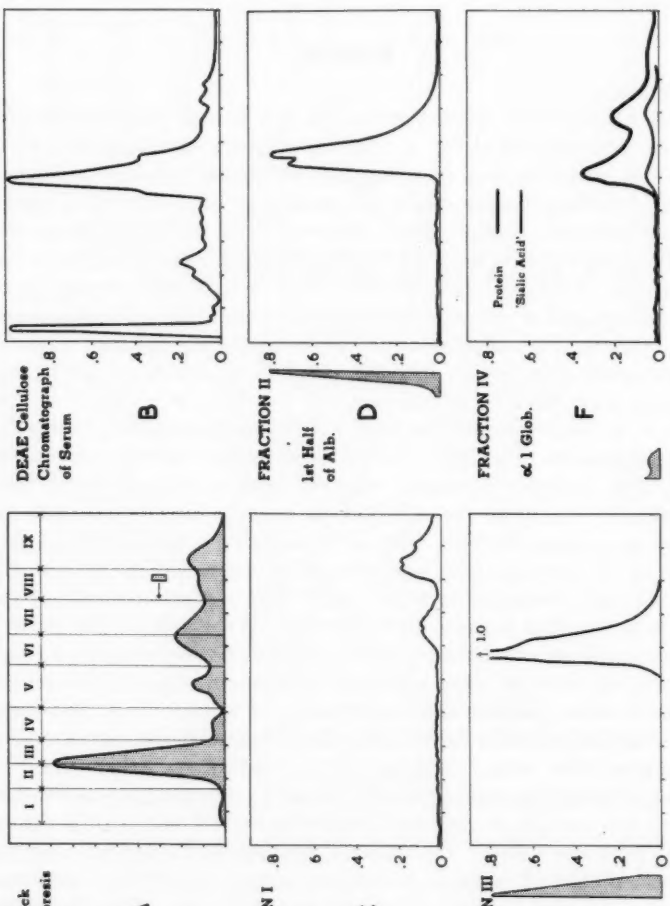
Starch Block Electrophoresis of Serum
I
II
III
IV
V
VI
VII
VIII
IX
A
DEAE Cellulose Chromatograph of Serum
B
FRACTION I
Pre Alb.
C
FRACTION II
1st Half of Alb.
D
FRACTION III
2nd Half of Alb.
E
FRACTION IV
α1 Glob.
F
Protein
Sialic Acid
1.0
.8
.6
.4
.2
0

FIG. 3, A–F.

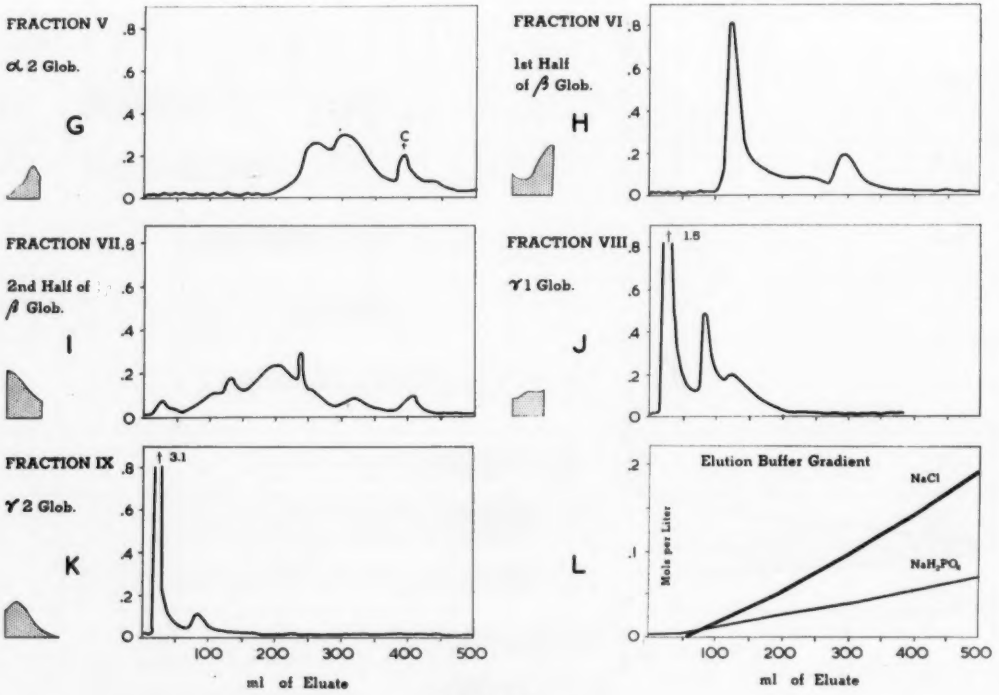

FIG. 3, G–L.

FIG. 3. The chromatography of serum electrophoretic fractions. Protein ——, "sialic" acid ——.

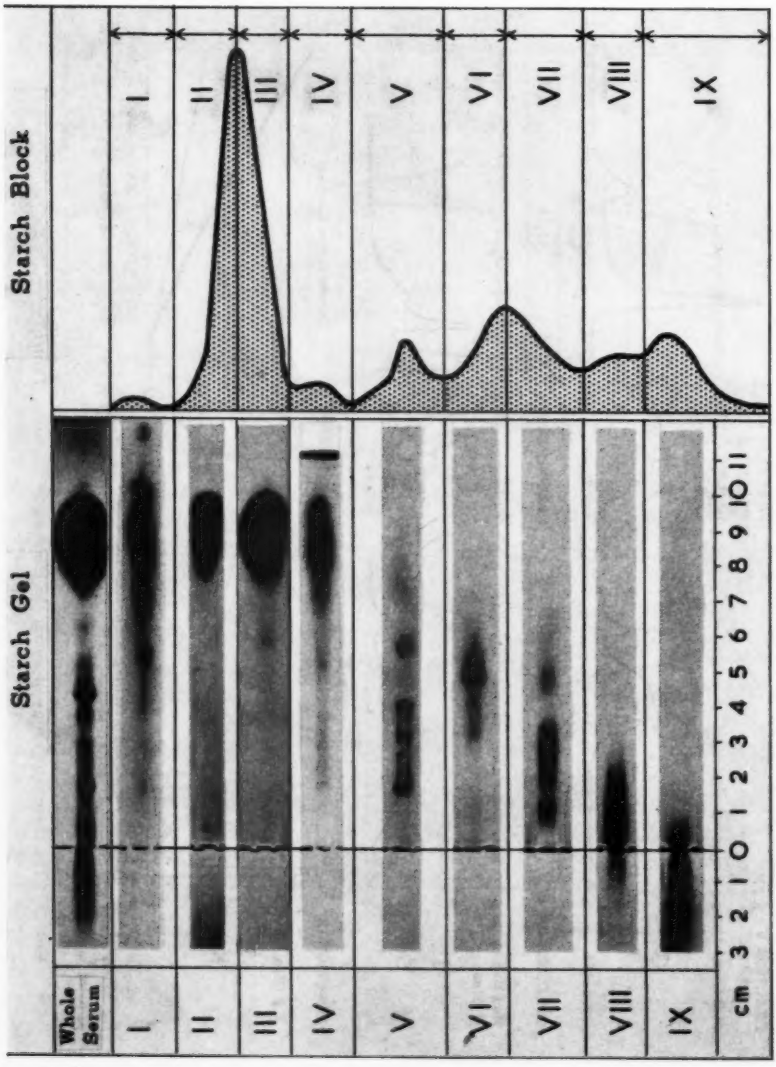

FIG. 4. Starch gel electrophoresis of serum fractions obtained by starch block electrophoresis.

of the starch block separation. It would appear therefore that this material has a similar charge to that carried by albumin, but has a smaller molecular size, so that the starch gel separates if from albumin by a molecular seiving effect. In addition, there is a small band at 5.8 cm. It is suspected that this may be a polymer of albumin.

The α_1 globulins constitute fraction IV. Chromatography on DEAE cellulose separated these into two components, each of which contained "sialic" acid. These were the only fractions in which a significant proportion of "sialic" acid was found. The smaller peak at 340 ml is the α_1 acid mucoprotein (orosomucoid) of Weimer, Mehl, and Winzler (24). The peak at 255 ml probably includes the M-2 fraction of Mehl, Golden, and Winzler (28).

The starch gel separation of fraction IV also showed two major components, with fronts at 10 and 8½ cm as well as some diffuse material at 7 cm and some traces of components seen in fraction V. The component at 10 cm was the prealbumin seen in the whole serum separation, proximal to the albumin, and is thought to be α_1 acid mucoprotein. The other component has the same mobility as albumin and has been identified by Poulick and Smithies as an α_1 globulin (12).

The DEAE cellulose partition of the α_2 globulin is shown in Fig. 3G. This fraction of the starch block was pink by transmitted light, probably due to hemoglobin, and green by reflected light, probably due to ceruloplasmin. There are four components to be seen in this pattern. While the first peak is at the same place as the first peak of the α_1 globulins, it does not represent an overlap in the starch block separation of the α_1 and α_2 globulins since no sialic acid was found in the α_2 fraction. Also, no component common to both fractions IV and V was observed in the starch gel separations. The peak marked "C" was identified as ceruloplasmin by its oxidase activity and by precipitation with ceruloplasmin antiserum.

The starch gel separation demonstrates the haptoglobins which are present in this fraction. Seven haptoglobin fractions are distinguishable between 1.6 and 4 cm. In addition, the ceruloplasmin fraction is seen at 5.8 cm.

The sixth starch block fraction, i.e. the first half of the β globulins, was a red color. Spectroscopic examination showed a broad absorption band in the green-blue but failed to reveal the characteristic double absorption bands of oxyhemoglobin. The color is probably due to transferrin. The DEAE cellulose chromatographic pattern is shown in Fig. 3H. It consists of an initial sharp peak at 125 ml of eluate, which falls to a plateau, then a final peak at 300 ml of eluate. The first sharp peak is probably transferrin since pure preparations of this protein chromatograph to this position. The last peak at 300 ml has the same position as the main peak of the preceding starch block fraction. However, it is probably not an overlap with this fraction since no more than a trace of duplication is discernible in the starch gel separations. In this separation (Fig. 4), the bulk of the protein is in a single heavy band, preceded and followed by more diffusely spread material.

The second half of the β globulins was colored yellow, probably due to carotene. The DEAE cellulose chromatograph (Fig. 3I) showed a series of seven peaks. The first of these, at 25 ml, is probably an overlap of the large main peak in the next starch block fraction. The third peak, at 125 ml, is also probably a carry-over from the main peak of the preceding fraction. In view of the fact that the two β fractions were separated at the peak of the β components, there is remarkably little duplication in the DEAE cellulose separations or in the starch gel separations. In the latter, there was indication of some carry-over from the main peak of the preceding β fraction at 5 cm. The main bulk of the material was present as a diffuse band with a more sharply defined band superimposed in the middle. There was also a sharp band at 1 cm that is in the area of the fast γ-globulin component.

The DEAE cellulose chromatograph of the fast γ fraction (Fig. 3J) shows three components. The largest part of the material appears in an initial peak which is neutral or cationic at pH 7.0 since it is not retained by the column at this pH. Speer *et al.* (10) were able to distinguish a small additional component in this peak by starting the column at pH 9.0. Two other peaks at 80 and 125 ml are also found. The starch gel electrophoresis shows only a broad diffuse band in this fraction.

The final starch block fraction of the γ globulins again shows mainly the initial peak at 25 ml on DEAE cellulose chromatography (Fig. 3K). There is some carry-over of the peak at 80 ml also, but no evidence of the peak at 125 ml of the previous γ fraction. The starch gel again shows a broad, diffuse band, but this time it is behind the origin.

Urine Proteins

A variety of urine protein chromatographs are shown in Fig. 5. A study of these, and similar chromatographs, and comparison with the serum protein fractionations, has shown that these patterns can be conveniently divided into nine zones.

Zone I is the first portion up to about 50 ml of eluate. It carries material that is neutral or cationic at pH 7 so that it is not retained by the column. The major fraction of serum γ globulin appears here. There is a prominent peak here in most urinary chromatographs.

Zone II is an area of low protein from about 50 to 100 ml. In serum a second γ globulin component appears here. Figure 5G is the chromatograph from a case of multiple myeloma in which the bulk of the protein appeared in this zone.

Zone III is a group of one to three prominent peaks usually seen in the 100- to 150-ml area. It is best illustrated in Figs. 5B, 5C, 5D, and 5E. Serum β protein has a sharp peak here.

Transferrin has been identified by immunochemical means as a major component of nephrotic urine protein in this zone. A component of serum γ globulin also appears here.

Zone IV is another valley although some small peaks may appear here. It

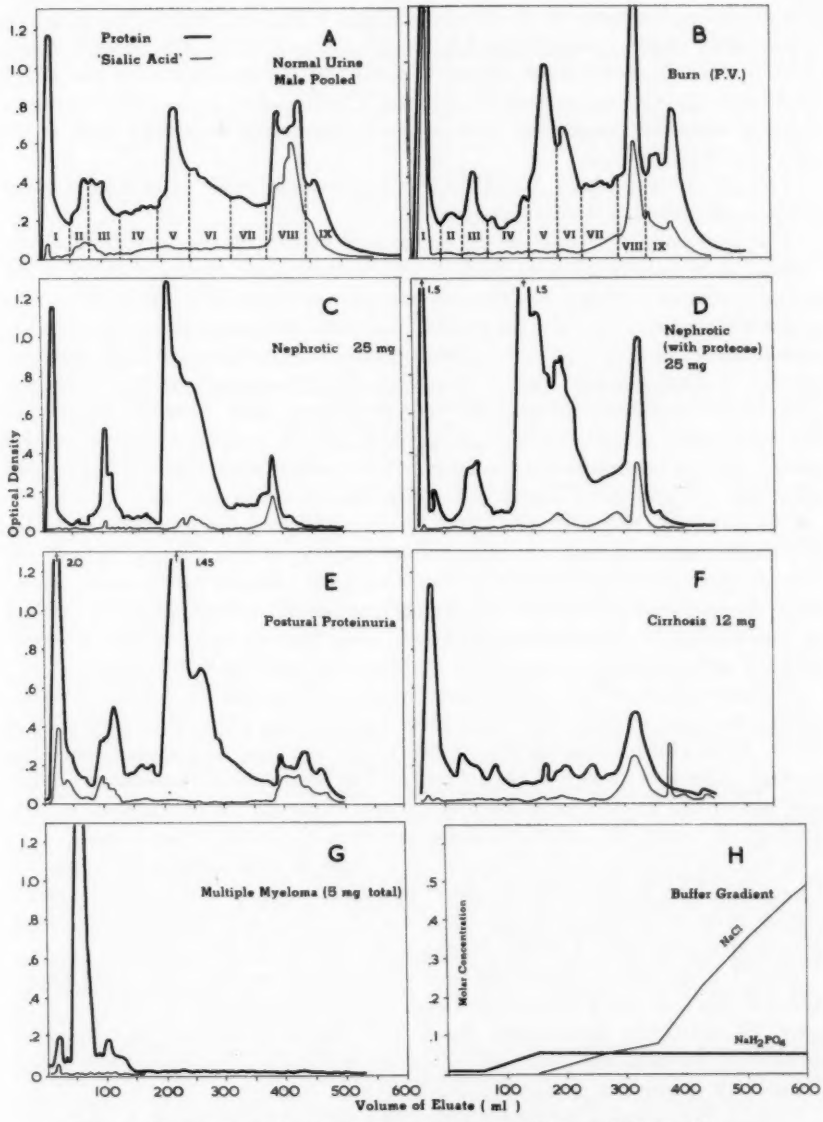

FIG. 5. Chromatographs of urinary proteins. Protein ——, "sialic" acid ——.

extends to the beginning of the next peak, which is usually the most prominent in the chromatograph. This large peak is an agglomeration of material which

usually takes the form of an initial high peak with a trailing decline in which a secondary peak appears (Figs. 5A, 5B, 5C, 5D, and 5E.) The peak is, therefore, divided into two zones. Zone V is the portion encompassing the initial high peak. It is composed largely of albumin, although α_1, α_2, and β components are also found here in serum. Zone VI encompasses the secondary peak in the albumin slope.

The region from about 280–350 ml comprises zone VII. It is a valley that seldom contains any distinctive peaks.

The next zone, VIII, is the region of acid mucoproteins. The bulk of the "sialic" acid usually appears here. In the normal adult, about 30–40% of the urinary protein is found in this and the next fraction (Fig. 5A). While the nephrotic excretes a smaller proportion of protein as mucoprotein, the absolute amounts may be large. In fact, nephrotic urine is a good source for the preparation of α_1 glycoprotein. Figure 5D shows the chromatograph of a nephrotic urine that contained the "proteose" of Levine *et al.* (29). It seems likely that the "proteose" is actually the mucoprotein seen in increased amount in this urine. The proteins found in the urine from a patient with cirrhosis of the liver (Fig. 5F) are composed largely of γ globulins and mucoproteins.

The last zone, IX, is usually a trailing area following the mucoprotein peak. We would expect to find acid mucopolysaccharides in this region. Indeed, the mucopolysacchrides excreted in Hurler's syndrome appear in this zone. However, the carbozole reaction of Dische (25), in the dilution in which we applied it, was unable to demonstrate hexuronic acids here in most urines. In some cases of severe burns, the carbozole reaction was positive in this region.

The evaluation of the urinary protein chromatograms from burn cases required that the information which they contained be reduced to a numerical basis. Each chromatograph was divided into the nine zones previously described. The area of each zone, divided by the total area of the chromatogram and multiplied by the total 24-hour protein excretion as determined by the method of Lowry (21), gave the daily excretion of protein in each zone.

A total of 19 urine specimens from five cases of severe burns are reported. In some cases, the specimen consisted of urine from 2 or 3 consecutive days, pooled prior to analysis. In no case was the specimen obtained later than 9 days after the burn. Usually, it was in the 1st to 6th day period.

Case I (J.S.) was a 6-year-old boy who suffered second- and third-degree burns to the face, neck, and upper chest. They covered 13% of the total body area. He stood the initial burn shock well. His total urinary proteins were never very elevated and returned to normal in 5 days. Most of the low values for urinary proteins in Table II came from this case.

Case II (S.C.) was a 3-year-old girl with a similar burn to case I. However, it was deeper and she had more reaction. Her urinary proteins were elevated to 3 or 5 times normal and remained high during the period of observation.

Case III (P.L.), a 5-year-old boy, had the most severe burn, 60% of his body being affected. He died 3 weeks after the burn. After the 1st day, when his

TABLE I

Percentage of the total protein in each chromatographic zone (Mean values and maximum range for 19 urines from five cases of severe burns)

Zone	Mean (%)	Range	Normal pooled urine
I	12.5	7.8–19.2	9.9
II	6.4	1.8–13	5.4
III	6.4	2.4–10	9.7
IV	5.0	2.8– 8.1	7.9
V	11.8	5.6–19.6	13.8
VI	8.7	3.3–14.7	14.3
VII	8.8	3.7–23.4	9.3
VIII	23.5	11 –32	21.9
IX	17.3	6.5–35	8.1

TABLE II

Protein excretion in each chromatographic zone as "mg albumin" (Mean values and maximum observed range for 19 urines from five cases of severe burns)

Zone	Mean	Range	Maximum normal
I	31	8 –120	9
II	15.9	1.8– 72	4.8
III	17.7	4.7– 62	8.7
IV	14.1	3 – 45	7.1
V	34.5	5.2–107	12.4
VI	21	3 – 52	13
VII	21.8	3.5– 58	8.4
VIII	49	14.8–120	19.7
IX	43.8	8 – 97	7.3
Total protein	282	57 –714	90

protein excretion was 7 times normal, it remained fairly constant at 3 times normal for the remainder of the observation period.

Case IV (C.B.), a 6-year-old girl, suffered flame burns to 33% of her body surface. She developed a persistent fever and pyogenic staphylococcus infection after 2 days. She died after 23 days. Her total protein excretion was near normal the 1st day but rose steadily until it was 5 times normal on the 6th day.

Case V (P.V.) was a 3-month-old boy who had a scald burn to 40% of his body around the head, face, chest, and arms. He did not appear toxic and had no fever until after 10 days. He died on the 12th day. His protein excretion was about 2–3 times the normal value (for older children) until the 8th day when it rose to 8 times the normal value.

The chromatogram (Fig. 5B) is that of the 1st day urine from this patient. Comparison with the normal chromatogram (Fig. 5A) illustrates some general variations of burn urines from the normal, although these were by no means consistent with all burn urines, as may be seen by the wide ranges shown in Table I where the mean proportion of total protein found in each zone for the 19 burn urines is given along with the values for the normal pooled urine. The proportion of the γ globulin fraction in zone I tends to be raised, while the proportions of β fractions in zone III are decreased. The large amount of

apparent "sialic" acid found coincident with the γ globulin proved to be due to dextran administered as a plasma expander. The color given by dextran with diphenylamine is bluer than that given by sialic acid. It had an absorption curve identical with that of the color from this fraction.

The next area of significant difference is zone VIII. Although there is no difference in the proportion of total protein in this zone, as seen in Table I, the peak in the burn urine chromatographs was consistently sharp and symmetrical compared with the rather heterogeneous peak in the normal. Hence the absolute increase in amount of mucoprotein excreted seems to be confined to a single material.

A major increase in the proportion of protein in zone IX is found in burns. This area contains acid mucoproteins or mucopolysaccharides. It is the only area in which hexuronic acids have been found. These have been found here in Hurler's syndrome and in early burn specimens where they may reach a concentration of 20 μg per ml of eluate.

The chromatographs and Table I are records of the relative composition of the urinary proteins. Table II shows the actual amount of the various protein fractions expressed as milligrams of equivalent albumin excreted per 24 hours. The total protein excretion ranged from normal to 8 times normal with a mean value of 3 times normal. This increase was found in varying degree in all areas of the chromatogram, the mean protein for each zone being higher than the normal maximum in every case. The most marked increases were in the γ globulin (zone I), and the acid mucoproteins and mucopolysaccharides (zones VIII and IX). In a few cases, the increases were largely confined to these zones so that the intervening zones of the chromatogram were low and indefinite, such as seen in cirrhosis of the liver (Fig. 5F).

Conclusions

The combination of starch block electrophoresis with DEAE cellulose chromatography has been found to have a high resolution. Some 22 serum proteins have been distinguished by this procedure. It has the added advantage that protein fractions may be prepared in considerable quantity for further study. The combination procedure has demonstrated the complexity some of the protein fractions obtained by chromatography alone, particularly in the 250 to 400 ml range where components from all electrophoretic fractions except the γ globulins are found. Despite this, the chromatographic resolution of proteins by the DEAE cellulose column can be an effective method of studying the urinary protein excretion. The difference in the chromatographs of the normal adult and the nephrotic show that the nephrotic excretes proteins that differ not only in amount, but in kind. The presence of "sialic acid-like" material in the early fractions of proteins excreted by the case of proteinuria (Fig. 3E), and the rather heterogeneous mucoprotein peak, distinguishes it from the nephrotic which it otherwise resembles. The increased excretion of mucoprotein along with γ globulins in cirrhosis (Fig. 5F) reflects the concurrent

increased production of both of these proteins.

While the protein excretion in burns was increased above normal, the increase is not enough to contribute significantly to any nitrogen loss such as has been reported in burn patients by others (13, 14). The increase in the first zone and the last two zones could be due to depolymerization products from the site of the burn. Gelatine, found by Rosenthal *et al.* (19) to be a product of scalded skin sections, appears in the first zone. Similarly, the increase in zones VIII and IX might be due to the mucoproteins, also shown by Rosenthal to arise from burned skin. Baar (30), using a charcoal adsorption procedure, reported a twofold increase in carbohydrate–polypeptide complexes in the urine of burn patients.

The general over-all increase in urinary proteins may be due to a large variety of abnormal proteins produced by the burn. On the other hand, they may represent leakage of plasma protein due to toxic effects on the kidney. The elucidation of this point requires further investigation.

We were unable to demonstrate any toxic materials in the proteins of burn urine. The total proteins prepared by ultrafiltration and dialysis were lypophilized, redissolved in a minimum of normal saline, and injected intraperitoneally into young mice. Injection of as much as 25 mg per mouse failed to produce any grossly apparent toxic effect.

Acknowledgments

We are particularly grateful for the competent technical assistance given by Mrs. Ingrida Gulbis and Mrs. Beverly Cushnie.

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Addendum

While the foregoing report was in press, a publication by Anderson, Lepper, and Winzler (1) came to hand. In this study normal urine colloids, collected by ultrafiltration, were fractionated on a DEAE cellulose column. A marked difference in the chromatograms of Anderson *et al.* and ours is at once apparent. Whereas the material in our chromatograms of normal urine colloids is separated into four, more or less evenly spaced, peak areas (Fig. 5A), the bulk of the proteinaceous material in the normal chromatogram of Anderson *et al.* (Fig. 1 (1)) lies in the first third of the chromatogram and in the last quarter of the chromatogram with very little in the intervening space. Some explanation or speculation concerning this difference is warranted.

While we both used ultrafiltration, the process differed in several important respects. Anderson *et al.* used collodian membranes capable of retaining molecules as small as 10,000 molecular weight. The urine colloids were concentrated until they formed a solid mat on the membrane. Only about one-quarter of this material was directly extractable into the starting buffer. The remainder was dissolved by dissolution of the membrane in alcohol and ether and solution in 0.1 *N* sodium hydroxide.

In contrast, we used pure cellulose tubing which would retain molecules above 20,000 molecular weight; the colloids were merely concentrated into a small volume of solution and not actually removed from solution; and we used only the material which remained in solution and was recovered from the ultrafiltration sack by washing with buffer. Therefore, the colloid sample obtained by Anderson *et al.* might differ from ours in several important respects. It may include smaller molecular weight material; it may include material lost in the walls of our cellulose sacs; and it may contain artifacts produced by the much harsher treatment. In respect to the last possibility, it has been our experience that these materials may be radically altered by alcohol precipitation at temperatures higher than $-5°$ C or by the use of strong alkali to affect solution of precipitates.

The pH gradient of the eluent used by Anderson *et al.* encompassed a much wider range (9.0–1.4) than ours (7.0–4.0). In fact, the last two-thirds of their chromatograms were eluted at pH's more acid than we attained at any point. It seems reasonable to expect that the material in our entire chromatogram would appear in the first third of the Anderson chromatogram. The fact that orosomucoid is found in our zone VIII near the end of our chromatogram

(Figs. 5A and 5B), but in Anderson's fractions six and seven, after only one-quarter of his total chromatogram is completed, confirms this. It is interesting to note that we recovered 80% of the material loaded on the column (as protein) whereas Anderson recovered only 38% (by weight) despite his more extensive elution.

We believe, therefore, that our urinary colloid preparation corresponds to Anderson's fraction A, i.e. the material extractable from his collodian membrane deposits by the starting buffer. Furthermore, it is this material that is more readily recovered from the column and appears in the first third of Anderson's chromatograms. The material in the latter two-thirds of the chromatogram, and his low recoveries, derive from his fraction B, or that material obtained by dissolution of the collodian membrane.

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EFFECT OF VARIOUS PREPARATIONS OF PITUITARY AND DIENCEPHALON ON THE IN VITRO SECRETION OF ALDOSTERONE AND CORTICOSTERONE BY THE RAT ADRENAL GLAND[1]

O. J. Lucis, I. Dyrenfurth, and E. H. Venning

Abstract

Purified corticotropin and ACTH peptides increased the secretion of aldosterone, corticosterone, and an unidentified compound RT_4 in incubated rat adrenal tissue. When the response was expressed as a percentage increase above that of the control tissue, the increases in corticosterone and compound RT_4 followed a sigmoid log dose – response curve. The maximum effect on aldosterone was obtained at a time when the response curve for corticosterone assumed a linear relationship between the response and the logarithm of the dose of ACTH. This dose level was considerably less than that required for maximal stimulation of corticosterone.

The capacity of the ACTH peptides $\alpha_1+\alpha_2$ and δ' for stimulating aldosterone secretion could be greatly diminished by allowing solutions of these fractions to stand at 5° C for 1 week. These solutions still retained their ability to stimulate corticosterone secretion.

Saline suspensions and extracts of fresh hog diencephalon contained a factor which selectively stimulated aldosterone secretion.

The present studies were designed to investigate the effect of various extracts of pituitary and diencephalon on the in vitro secretion of aldosterone, corticosterone, and an unidentified compound RT_4. The anterior pituitary extracts included USP standard ACTH, hog and human ACTH, and various ACTH peptides. A few commercial posterior pituitary and pineal preparations were also examined. The adrenal of the rat secretes five ultraviolet-absorbing compounds (1, 2). Corticosterone and compound RT_4 comprise about 75% of the total ultraviolet absorption, while aldosterone contributes approximately 15% and the remainder is derived from the two polar compounds RT_1 and RT_2.

Methods

The method of Saffran and Schally (3) was used for studying the effect of corticotropin on the in vitro secretion of corticosteroids. Forty hooded male rats weighing 180 ± 10 g were usually used for each test. The rats were anesthetized with sodium pentobarbitone and the adrenals were excised, dissected free from fat, and placed on a petri dish which was kept cold with crushed ice. The adrenals were covered with filter paper moistened in Krebs–Ringer solution until all the adrenalectomies had been completed. The adrenals were then cut into four equal portions and a quarter of each gland was placed in each of four

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separate beakers containing cold Krebs–Ringer bicarbonate glucose solution (KRG) (4). The tissue was blotted on filter paper and weighed to the nearest milligram. When 40 rats were used the weight of the adrenal tissue in each beaker averaged 0.260 g. In a few experiments where a larger group of rats was used the weight of the tissue in each beaker was proportionately greater. In every case the volume of the incubation medium (KRG) was maintained at 30 ml per sample.

The four portions of adrenal tissue were incubated for 1 hour in a Dubnoff metabolic shaking incubator,* the medium was then decanted and discarded, and fresh KRG solution was added. One of the aliquots of adrenal tissue was used as a control; to the other three, varying amounts of the substances to be tested were added. These were dissolved in 30 ml KRG solution. The tissue was incubated for an additional 2 hours. At the end of this period, the steroids were extracted from the incubation medium with chloroform. After evaporation *in vacuo* a crude extract was obtained. In a few experiments rat adrenal capsules and decapsulated adrenal glands, obtained by the method described by Giroud *et al.* (2), were incubated in a similar manner.

The following paper chromatographic systems were used for the separation of the steroids in the crude extract: chloroform/formamide (5), toluene/methanol/water (6), toluene/propylene glycol (7), benzene/methanol/water (6).

An initial separation of aldosterone from the other ultraviolet-absorbing steroids could be obtained in either the chloroform/formamide system or the Bush B_5 system (benzene/methanol/water). The aldosterone fraction was eluted and rerun in the systems Buch C (toluene/methanol/water) and Bush B_5. The fraction containing RT_4 and corticosterone was rerun in the toluene/propylene glycol system where the two steroids were readily separated. Aldosterone, corticosterone, and RT_4 were eluted from the paper strips and were measured in an ethanolic solution by ultraviolet light absorption using a molar extinction coefficient of 15,800 for aldosterone and 16,700 for corticosterone. An assumed molar extinction coefficient of 15,800 and a molecular weight of 350 were used in the calculation of the amount of RT_4. Aldosterone and corticosterone were also measured by the color reactions with blue tetrazolium (8) and isonicotinic acid hydrazide (9). The discrepancies between corresponding results obtained by these methods were less than 10%.

The variation in the in vitro secretion rate of aldosterone and corticosterone in the four portions of the quartered adrenal tissue was investigated; the maximum deviation from the mean was $\pm 8.3\%$ for aldosterone and $\pm 8.7\%$ for corticosterone (10). There was, however, some variation in the rate of secretion of these two hormones in different groups of rats depending to some extent upon the release of endogenous corticotropin during the surgical procedure. In order to assemble data obtained on different days, the response of the adrenal tissue to the addition of various stimulating substances was expressed as follows:

*Manufactured by Precision Scientific Company.

$$\frac{\text{increment in secretion rate}}{\text{control secretion rate}} \times 100.$$

This will be referred to as percentage increase above that of the control value. This manner of expression reflects the relative change in adrenal activity and repeated assays showed that the response of corticosterone was reasonably well reproducible.

Results

The chromatographic separation of the compounds present in the incubation medium of rat adrenals confirmed the presence of five ultraviolet-absorbing compounds as shown in Fig. 1. These consisted of two polar compounds RT_1

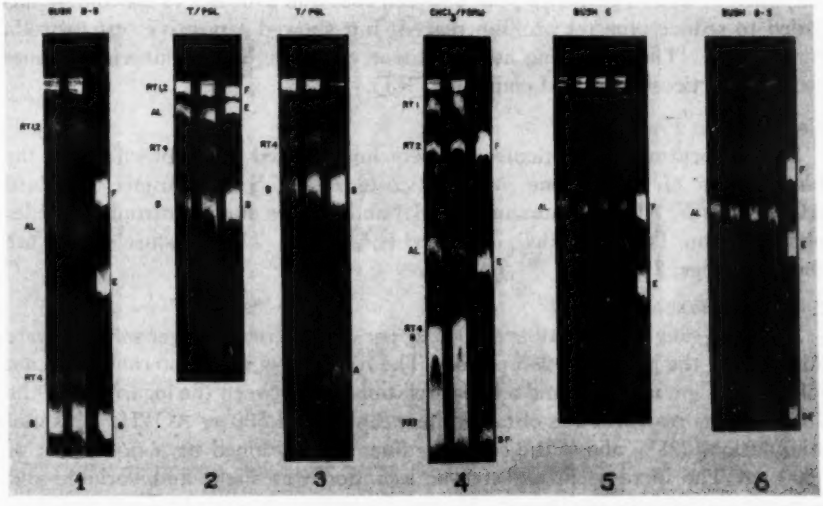

FIG. 1. Chromatographic separation of steroid hormones elaborated by the adrenal glands of rats. The distribution of the five ultraviolet-absorbing compounds are shown in the systems (1) Bush B_5, (2) toluene/propylene glycol, (4) chloroform/formamide. In the Bush C system (5) aldosterone has the same mobility as hydrocortisone, while in Bush B_5 system (6) aldosterone runs between hydrocortisone and cortisone. RT_4 and corticosterone were separated in the toluene/propylene glycol system (3).

and RT_2, aldosterone, RT_4, and corticosterone. Aldosterone was characterized by its chromatographic mobility in the following systems: chloroform/formamide, toluene/propylene glycol, Bush C and Bush B_5, and also in the E_2B system of Eberlein and Bongiovanni (11). In concentrated sulphuric acid this isolated material gave the expected absorption maximum at 2880 Å and in ethanolic solution it absorbed ultraviolet light maximally between 2390 and 2400 Å. This substance reacted quantitatively with blue tetrazolium reagent and, in the bio-assay of Venning *et al.* (12), showed a potency comparable to

authentic aldosterone. Corticosterone was characterized by its chromatographic mobility in three different systems: chloroform/formamide, toluene/propylene glycol, and Bush B_6, by ultraviolet absorption and reduction by blue tetrazolium. Its spectrum in concentrated sulphuric acid showed peaks at 2850, 3300, 3750, and 4550 Å.

When the adrenal glands were decapsulated, the capsule consisted mainly of the zona glomerulosa as previously shown by Giroud *et al.* (2). The possibility that small amounts of zona fasciculata might still be present in these adrenal capsule preparations was not excluded. Incubation of the capsules showed that this tissue secreted mainly aldosterone with small amounts of RT_1, RT_2, and corticosterone. In ethanolic solution the maximal ultraviolet light absorption for RT_1 and RT_2 was 2400 Å, and both compounds showed, in concentrated sulphuric acid, a single sharp peak between 2880 and 2900 Å. These compounds failed to reduce blue tetrazolium reagent but showed a positive test for soda fluorescence. The remaining adrenal tissue consisting of the innermost zones secreted corticosterone and compound RT_4.

Corticotropin Preparations

Six preparations of corticotropin were investigated for their effect on the biosynthesis of aldosterone and corticosterone: U.S.P. reference standard ACTH, Nordic ACTH,* human ACTH,† and a series of corticotropin peptides obtained from Dr. P. H. Bell, $\alpha_1+\alpha_2$, $\gamma^1+\gamma^2$, and δ'. The response curves are shown in Figs. 2 and 3.

U.S.P. Standard ACTH

Amounts ranging from 10 to 10,000 μg per 30 ml Krebs–Ringer solution were added after the preincubation period. The response in corticosterone secretion followed a sigmoid curve and a linear relationship between the logarithm of the dose and the response was obtained between 40 and 500 μg ACTH. Maximal stimulation, 225% above the control value, was obtained at a dose level of 1000 μg. The increase in aldosterone secretion was slight and variable and was not statistically significant. Maximal stimulation was obtained at a dose level of 50 μg ACTH.

Nordic ACTH, Lot No. A-6502

This material prepared from hog pituitaries by oxycellulose adsorption techniques was a highly purified commercial corticotropin. It was added in amounts ranging from 0.05 to 505 μg per 30 ml. The response for corticosterone secretion showed a linear relationship between dose levels of 4 and 100 μg ACTH. When 505 μg were added, corticosterone was increased 400% above the control value. The response in the secretion rate of aldosterone was more variable than that observed for corticosterone. Maximum responses occurred between the dose levels of 5 and 10 μg. ACTH and these were statistically

*This preparation was kindly supplied by Mr. K. Antoft of Nordic Biochemicals, Ltd., Montreal, Que.

†Human ACTH was prepared from post-mortem material by the procedure of Dr. Raben.

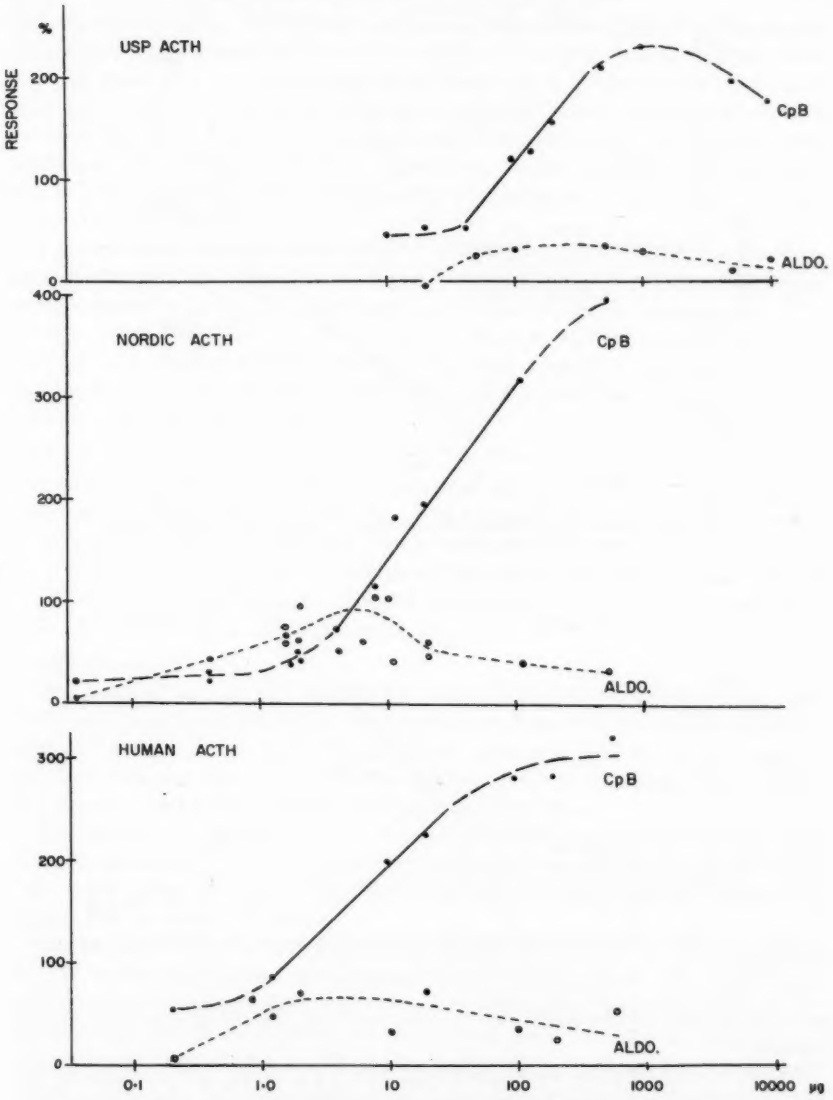

FIG. 2. Effect of various preparations of corticotropin on steroid formation by rat adrenal quarters in vitro.

significant at dose levels between 0.4 and 20 μg ($p < 0.001$). At lower dose levels of Nordic ACTH the response in aldosterone secretion was slightly greater than that observed for corticosterone. However, with higher doses the

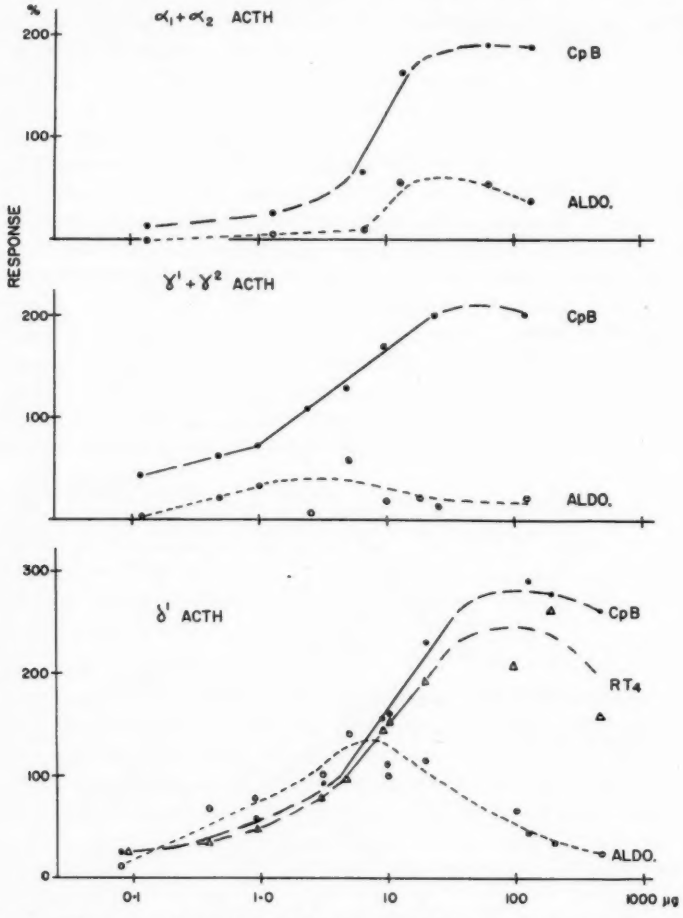

FIG. 3. Effect of corticotropin peptides on steroid formation by rat adrenal quarters in vitro.

response of aldosterone decreased in contrast with the rising levels of corticosterone.

Whole adrenal glands were separated into two parts, one containing the capsule consisting mainly of the zona glomerulosa and the decapsulated part containing the remaining zones. Nordic ACTH, added in amounts ranging from 0.4 to 20 μg to the capsular tissue, caused increases in aldosterone secretion ranging from 78 to 135% above the control values. A rise in corticosterone secretion also occurred in this tissue but the response did not show a linear relationship to the logarithm of the dose of ACTH. Compounds RT_1 and RT_2

were secreted only by the capsular tissue and were not observed on the paper strip when decapsulated tissue was incubated. While no measurements were made of these two ultraviolet-absorbing compounds, visual observation indicated that these compounds were present in greater amounts when aldosterone secretion was enhanced. The decapsulated tissue secreted corticosterone and compound RT_1. When ACTH was added a significant increase in the secretion rate of corticosterone and the compound RT_4 occurred. No aldosterone was secreted by this tissue.

A preliminary attempt was made to resolve the aldosterone and corticosterone stimulating properties of Nordic corticotropin by polypeptide paper chromatography in the *n*-butanol:acetic acid:water (4:1:5) system (13). The material separated into several ninhydrin reactive zones. Incubation of rat adrenal with these fractions revealed that each caused an increase in aldosterone secretion with a still greater effect on the secretion of corticosterone. Further work was not carried out.

Human ACTH

Human corticotropin (HOMO 2), prepared by Dr. M. Raben, was added in doses ranging from 0.17 to 570 μg. The response in the secretion rate of corticosterone showed a linear log dose relationship between the doses of 1 and 30 μg of ACTH. The aldosterone response was again more variable than that of corticosterone. The increases observed with doses of 0.95–1.7 μg ranged from 35 to 72% above that of the control value.

ACTH Peptides

The fraction $\alpha_1+\alpha_2$ (lot S-1079-31) was added in amounts ranging from 0.14 to 140 μg. An increase in aldosterone secretion was not observed until a dose of 14 μg was reached. Between this dose and 140 μg the increase in secretion rate was significant. Corticosterone secretion increased more rapidly and showed a maximal response at a dose level of 50 μg.

The fraction $\gamma^1+\gamma^2$ (lot S-1079-1) was added in amounts similar to those used in the previous experiment. The response of corticosterone showed a linear log dose relationship between 1 and 30 μg of this fraction. The aldosterone response was, however, variable.

The fraction δ' (lot S-1079-24) showed properties similar to those of Nordic ACTH in the stimulation of corticosterone and aldosterone. At higher dose levels, however, the secretion of corticosterone tended to decrease. This ACTH peptide had the greatest effect on aldosterone secretion and a maximum response was obtained at 5 μg. With doses greater than 20 μg the response decreased and was not significant at 500 μg. The secretion rate of RT_4 was estimated in these experiments and the response curve showed a similar trend to that of corticosterone.

Effect of Small Amounts of Corticosterone on Aldosterone Secretion

In order to determine whether aldosterone secretion was influenced by the changing concentration of corticosterone in the incubation medium following

the addition of ACTH this steroid was added in amounts of 49.5, 99.0, and 198.0 μg per 30 ml KRG solution, and incubated with adrenal tissue. In these concentrations corticosterone did not alter the secretion rate of aldosterone. The mean secretion rate by the control tissue was 14.1 μg/g/hour and with added corticosterone, 14.0, 13.9, and 14.3 μg/g/hour. Increases in the secretion of corticosterone due to the addition of ACTH rarely exceeded 100 μg.

Alterations in ACTH Activity on Storage

When corticotropin $\alpha_1+\alpha_2$ was kept at +5° C in non-sterile Krebs–Ringer solution for 1 week, its ability to stimulate the secretion of aldosterone was greatly diminished while its capacity to increase the secretion of corticosterone was unchanged or increased. The results are shown in Table I. When 70 μg

TABLE I

Variability in response of fresh solutions of ACTH peptides and those stored for 1 week

ACTH peptides	Dose (μg)	Response in aldosterone secretion rate		Response in corticosterone secretion rate	
		Fresh ACTH % increase above control	Stored ACTH % increase above control	Fresh ACTH % increase above control	Stored ACTH % increase above control
$\alpha_1+\alpha_2$	11.2	—	8.6	—	270
	14.0	57.5	—	115	—
	29.4	—	6.5	—	380
	70.0	58.4	0	193	210
	140.0	39.4	—	193	—
δ′	3.2	103	—	95	—
	4.8	143	11.3	100	118
	9.6	109	—	155	—
Mixture $\alpha_1+\alpha_2$ +δ′	70.0 4.8	—	0	—	200

of a freshly prepared solution of $\alpha_1+\alpha_2$ ACTH was added the secretion rate of aldosterone was increased 58.4% above that of the control tissue. After 1 week of storage a similar dose had no effect. The effect on corticosterone secretion was unaltered. Similar observations were made with δ′ ACTH and with a mixture of these two peptides.

Presence of an Aldosterone-Stimulating Factor in Crude Posterior Pituitary Extracts

Giroud *et al.* (14) have previously observed that a crude commercial extract of posterior pituitary, Infundin, caused a selective increase in aldosterone secretion in incubated whole beef adrenal tissue while purified posterior pituitary hormones were without effect. A preparation of Infundin, lot S-035,* when incubated with quartered adrenals of rats in amounts equivalent to 1, 5, and 10 posterior pituitary units (200, 1000, and 2000 μg of dry weight), caused a significant increase in aldosterone secretion without affecting corticosterone secretion to any extent (Table II). Another batch of this preparation, lot V-123,* stimulated both aldosterone and corticosterone, suggesting the presence

*Burroughs Wellcome & Co.

of adrenocorticotropin. This material, in amounts ranging from 500 to 10,000 μg, caused an increasing response in the secretion of aldosterone and corticosterone.

TABLE II

Effect of commercial preparations of posterior pituitary tissue on the in vitro secretion of aldosterone and corticosterone

	Tissue weight (g)	Material added (μg)	Aldosterone (μg/g/hr)	Corticosterone (μg/g/hr)
Infundin V-123	0.288	0	12.3	33.4
	0.284	100	10.2	39.6
	0.288	500	15.6	46.6
	0.286	1,000	19.1	66.2
	0.280	0	12.3	32.4
	0.258	2,000	19.2	79.5
	0.282	5,000	20.4	103.7
	0.272	10,000	22.6	126.0
Infundin S-035	0.780	0	4.5	19.8
	0.808	200	7.7	21.4
	0.860	1,000	9.2	21.8
	0.770	2,000	10.0	19.5
Protopituitrin	0.280	0	13.0	25.0
	0.248	890	17.3	50.0
	0.254	1,780	18.8	49.0
	0.262	4,550	22.5	59.4

Another commercial posterior extract, Protopituitrin,* containing 12 to 30 units of pressor activity and 11 to 15 units of oxytocic activity per mg was investigated. At dose levels between 9 and 270 μg little effect on aldosterone and corticosterone secretion was observed. At higher dose levels, 890 to 4550 μg, both aldosterone and corticosterone secretion was enhanced (Table II).

A dried powder of ox posterior pituitary tissue, which was used for the preparation of Infundin V-123, was extracted with glacial acetic acid and precipitated with acetone. The yield of the dry precipitate was 11.3% of the original material. Various amounts of this precipitate dissolved in 0.5 ml of 0.5% acetic acid were added to the incubation medium of quartered whole adrenals, adrenal capsules, and the decapsulated adrenals. Similar amounts of acetic acid were added to the control tissue. Both aldosterone and corticosterone secretion were stimulated in the whole adrenal and in the capsule; the response of corticosterone secretion in the capsule, however, was considerably lower (Table III). No aldosterone was secreted by the decapsulated glands and the corticosterone response was also less than that observed for the whole gland.

These studies indicate that some commercial preparations of posterior pituitary contain contaminants which stimulate the biosynthesis of corticosteroids.

Diencephalon Tissue

Diencephalon tissue, including the pineal gland, was carefully dissected from

*Parke Davis & Co. Studies by Dr. M. Saffran showed that it contained 0.25 I.U. ACTH activity and some corticotropin-releasing activity.

TABLE III

Response of various zones of the rat adrenal to posterior pituitary extract

Tissue	Weight (g)	Amount of acetone precipitate (μg)	Aldosterone (μg/g/hr)	Corticosterone (μg/g/hr)
Adrenal	0.285	0	11.8	25.4
	0.258	9.5	13.9	74.4
	0.286	95	14.2	76.1
	0.276	475	18.0	103.0
	0.248	0	11.9	26.2
	0.240	950	19.2	95.0
	0.252	2,850	17.7	96.0
	0.234	9,500	15.8	81.0
Capsule	0.126	0	8.7	7.2
	0.124	11	12.1	12.1
	0.122	111	18.5	17.6
	0.132	555	14.8	14.0
Decapsulated adrenal	0.490	0	0	18.9
	0.500	555	0	32.5

TABLE IV

Effect of fresh hog diencephalon and beef pineal preparation on steroid formation by rat adrenal glands in vitro

Preparation	Material added (mg)	Weight adrenal tissue (g)	Aldosterone		Corticosterone (μg/g/hr)
			μg/g/hr	Response %	
Fresh hog diencephalon	0	0.286	6.1	—	28.8
	0.065	0.292	7.0	14.8	25.6
	0.650	0.262	9.8	60.8	27.7
	6.50	0.272	10.9	78.8	26.0
	0	0.330	4.8	—	20.0
	0.65	0.300	7.5	56.3	21.5
	6.50	0.314	9.0	87.5	22.4
	19.50	0.328	10.2	112	19.1
Pineal powder[a] No. 103351	0	0.250	5.0	—	35.0
	0.030	0.244	5.1	0	26.6
	0.300	0.250	4.6	0	27.0
	1.300	0.238	6.2	0	27.5

[a]Wilson Laboratories, Chicago, Ill.

freshly killed hogs. The tissue was immediately frozen and homogenized in the cold in a Waring blendor with 2 parts of saline solution according to the method of Farrell *et al.* (15). The saline suspension was refrozen until the assay was carried out.* Quartered rat adrenals were incubated with the saline suspension and also with the saline supernatant obtained after centrifugation. The dry weight of 1 ml of the suspension was 65 mg and that of the supernatant was 11.2 mg. Both the suspension of diencephalon tissue and the saline supernatant in doses from 0.10 to 3.0 ml increased the secretion of aldosterone without increasing that of corticosterone. The values obtained for the suspension are shown in Table IV.

*This tissue suspension was kindly prepared by Mr. K. Antoft, Nordic Biochemicals, Ltd., Montreal, Que.

Pineal Extracts

Two preparations of pineal body,* pineal powder No. 103351 and pineal extract No. 11, in amounts ranging from 7.3 μg to 1300 μg failed to affect the secretion of aldosterone or corticosterone (Table IV). Another pineal fraction B† and a melotonin concentrate† were inactive in this respect.

Discussion

Many conflicting results have been reported regarding the effect of corticotropin upon aldosterone secretion and the present studies show that the response of aldosterone using an in vitro bio-assay is more variable than that of corticosterone with different preparations of corticotropin and not well correlated to the dose of either corticotropin or the peptide fractions. In general the maximum effect on the biosynthesis of aldosterone occurred at a time when the response curve for corticosterone changed into the phase of linear log dose relationship and with increasing doses of corticotropin the response decreased. The response of corticosterone followed a sigmoid log dose – response curve as previously shown by Saffran and Schally (3) for the total ultraviolet-absorbing compounds secreted by the rat adrenal, while that of the unidentified compound RT_4 with ∂′ ACTH was similar to that of corticosterone. RT_4 appears to have the same characteristics as compound X_2 of Peron (16) and compound Y of Reif and Longwell (17). Studies with labelled precursors, desoxycorticosterone and progesterone, show that these steroids are converted to RT_4 by the rat adrenal tissue. Similar observations have been made in our laboratory with progesterone $4C^{14}$. Birmingham and Kurlents (18) have previously shown that compound RT_4 is stimulated by corticotropin.

In the present studies aldosterone secretion was rarely increased beyond 125% of that of the control tissue regardless of the preparation of corticotropin used, while the secretion of corticosterone continued to increase. This suggests that the limiting factor in the response to corticotropin may be a saturation of the enzyme systems involved in the biosynthesis of aldosterone and a shift in the biosynthesis pathways leading to greater formation of corticosterone and compound RT_4. These findings are in agreement with previous results obtained in man (19) where aldosterone excretion was first enhanced by ACTH and then decreased as the administration of ACTH was continued.

The ability of the corticotropin peptides to stimulate aldosterone appeared to reside in a labile factor which could easily be destroyed without affecting their capacity to stimulate corticosterone secretion and may well account for some of the variable reports on the activity of these fractions. That there is a species difference in the response to these peptides is borne out by the results of Farrell *et al.* (20) in decerebrate dogs where ∂′ ACTH was found to be several times more active than β-ACTH in the stimulation of aldosterone while Stachenko *et al.* (21, 22) were unable to obtain any significant effect of ∂′ and

*Wilson Laboratories, Chicago, Illinois.
†Preparations of Dr. A. B. Lerner, Yale University.

$\alpha_1+\alpha_2$ peptides on aldosterone synthesis in beef adrenal slices consisting mainly of the glomerulosa.

There is considerable evidence to indicate that aldosterone is primarily controlled by some factor other than corticotropin. The origin of this substance has still not been clearly established. Farrell (15, 20) proposed that the diencephalon or pineal body may be involved in the regulation of aldosterone, while the more recent work of Davis (24) suggests a renal control. A humoral control of aldosterone has been demonstrated by Yankopoulos and co-workers (25) in cross-circulation experiments. Our investigations show that a substance exists in fresh diencephalon which selectively stimulates the in vitro biosynthesis of aldosterone in the rat adrenal. A similar effect was observed with a commercial preparation of posterior pituitary, and confirms the earlier findings of Giroud *et al.* (14) using beef adrenal slices. These investigators showed that this effect was not due to vasopressin or oxytocin.

The present study adds further support for the existence of at least two trophic factors of different origin which influence aldosterone biosynthesis.

Acknowledgments

The authors wish to thank Dr. J. S. L. Browne for his support and advice. We are greatly indebted to Mr. K. Antoft, Nordic Biochemicals Limited, Montreal, for the donation of corticotropin and the preparation of hog diencephalon extract, and to Dr. P. H. Bell, American Cyanamide Company, for the donation of ACTH peptides. The technical assistance of Mrs. M. Boisjoli is gratefully acknowledged.

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Addendum

The compound RT_4 was recently characterized as 20 → 18-hemiketal of 18-hydroxy-11-desoxycorticosterone by M. K. Birmingham and P. J. Ward. (J. Biol. Chem. 1961. In press.)

COMPARATIVE EXAMINATIONS OF THE PHYSICAL CHARACTER OF GROUND SUBSTANCE BY MEANS OF GELATINOUS BULLAE[1]

MICHAEL C. HALL

Abstract

Fluid injected into the subcutaneous tissue of a rat will form in it a gelatinous bulla. This bulla can be excised intact, and comparative measurements of the rate at which the injectant is lost from it can be used to give information in regard to the character of the ground substance, or the effect of the injectant upon the ground substance. The rate of water loss was found to be decreased by advance in age, and by castration of the male. It was increased by hyaluronidase, by variations above and below pH 7, by maintenance on a subscorbutic diet. It was found greater with high temperatures than with low. The experimental results have been correlated with the known electrochemical nature of the ground substance, suggesting that the use of this method of bullae formation is a simple means by which comparative examinations of ground substance can be made.

Introduction

Connective tissue, the supporting tissue of the body, is divided for descriptive convenience into formed and amorphous components. The latter, known as the ground substance, is the milieu intérieur of the body, and is now regarded as "functioning as a coherent unit" (1). It is defined by Gersh (2) as including "all the non-fibrillar extra-cellular components of connective tissue". The ground substance contains mucopolysaccharides and proteins (3). The latter are both the soluble molecules of formation of developing collagen before they become part of the formed fibrous system, and a protein, which is not collagen, that complexes with the mucopolysaccharides (4). The protein of the complex is believed to play an important part in the viscosity and water-binding effect of the mucopolysaccharides (5, 6, 7, 8, 9). The protein and mucopolysaccharide have ionizing groups (10, 11) and Joseph *et al.* (11) state that "the physicochemical properties of connective tissue depend on the organization of the tissue matrix as a heterogeneous colloid". Histochemical examinations (12, 13, 2, 14, 15, 16, 17, 18, 19) and electrochemical titrations (20, 21, 22, 23, 11, 24, 25, 26, 27) have related the degree of polymerization to the water content and in general terms it has been found that highly polymerized ground substance contains less water than poorly polymerized ground substance, and that, when the water content has been varied by a physiological mechanism, the state of polymerization has also been varied. The method by which water is held in the tissue is not well understood, but from dye injection studies there appears to be no truly "free" water, only exchangeable water (28). Apart from the minimal essential water of hydration of the micellae, it is possible that the water of the ground substance is in successive shells around the charged micellae which exert an influence on the dipolar water molecules proportionate

[1]Manuscript received January 5, 1961.
Contribution from the Department of Anatomy, University of Toronto, Toronto, Ontario.

to their distance from each other (10). The experiments performed were based on the forced injection of water into loose connective tissue forming in it a bulla. The ability of this bulla to retain the injected water, when compressed, was examined and compared with a control. Such bullae have been used to demonstrate the substance of connective tissue (29, 30), but previous use in estimating water binding by connective tissue is not known.

Method

Male albino rats were used except in vitamin C deficient studies where male guinea pigs were used. There were five animals in each group. The procedures were carried out under light ether anaesthesia, and particular care was taken to prevent the degree of anaesthesia from becoming too deep, with consequent reduction of circulation to the skin. A flap of central abdominal pelt was turned back from the underlying muscle, exposing the translucent areolar fascia on its undersurface. A bulla was made in this areolar tissue by injecting normal saline into it from a hypodermic syringe (Fig. 1). A gauge 25 needle was used; 3 to 5 ml was injected, depending on the experiment, and the injections were performed in 30 to 40 seconds. The bulla was excised immediately from the surrounding skin and weighed. It was reweighed every minute and allowed to dry on filter paper between weighings. Between each measurement a weight was allowed to stand on the bulla for a constant length of time; this increased the rate of fluid loss from the bulla but was a constant factor in each experiment. It was found convenient to place the bulla on a perforated metal plate, covered by a filter paper, permitting free drainage. The perforated plate was in the bottom of a glass beaker, and another beaker containing mercury to weigh 200 g was set gently inside this, on the bulla, for exactly 15 seconds every minute. The weighings were discontinued after 10 minutes when the differences between them were becoming small. Since a bulla left in the atmosphere will eventually dry, the point at which variations in weight are difficult to appreciate represents only a slowing in the water liberation, and not the completion of the process. This final weight, however, represents the sum of two factors, the weight of the tissue proper and the weight of the remaining water with which it is reluctant to part. In order to separate these factors, and eliminate the variable of the weight of the tissue excised, the remaining tissue was crushed firmly in the fingers between filter paper and was weighed again. This seemingly oversimple method was believed adequate for these preliminary experiments in which widely separate variables had been chosen. The final weight was then subtracted from the weights taken minute by minute, converting the readings to weights of water remaining in the tissues, as shown in the example below.

Weighings		Calculated water content (g)
Initial weight (g)	2.13−0.32	=1.81
Weight (g) at 5 min	1.00−0.32	=0.68
Weight (g) at 10 min	0.75−0.32	=0.43
Final weight (g)	0.63−0.32	=0.31
Weight (g) of crushed bulla	0.32	

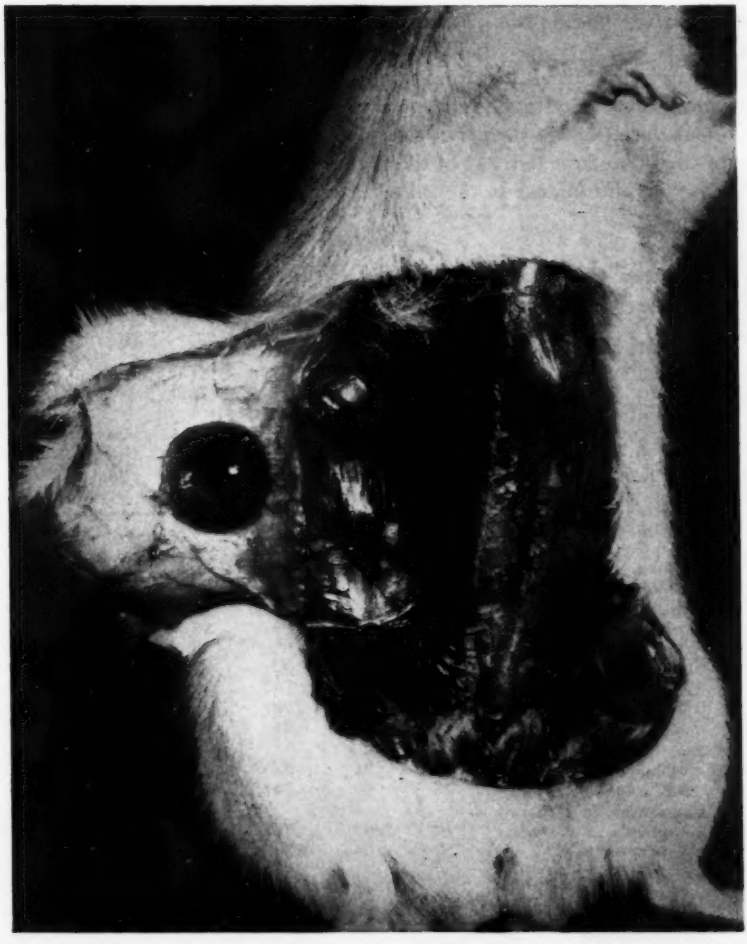


FIG. 1. Bulla *in situ* in subcutaneous tissue. Colored solution injected for photographic purposes.

The values for the five animals of the group were then averaged for each minute, and the average percentage of injected saline that the bulla had lost was plotted against time. In order to obtain a mathematical expression of the rate of water loss, which represents the rate of flow through the connective tissue, a logarithmic plot was constructed of log $[a/(a-x)]$ against time where x is the percentage weight of saline lost in t minutes, a is the maximum amount of saline lost, represented by the plateau of the extrapolated curve of the first graph. With relatively rapid loss of fluid caused by compressing the bullae, there was

no difficulty in finding this plateau. From this plot was developed a velocity constant k, which represents log $[a/(a-x)]$ per minute, and is an expression of the rate at which fluid is lost from the bulla.

Examinations were made of the comparative effects of different weights on the tissue, variations of the pH of the saline injected, variations of the temperature of the saline injected, age of the animal, castration in the male, vitamin C deficiency, hyaluronidase in the saline injected.

Results

Table I and the graphs, Figs. 2–8, are to some extent self-explanatory. Table II indicates in more detail one area of the experiments giving the scatter of the data.

TABLE I

The loss of water after compression of the bulla and the calculated velocity constants

Effect of:	% injected water lost at: 2 min	5 min	7 min	a	Velocity constant k
Weight application					
No weight	12	28	34	50	0.07
200 g	44	71	80	88	0.16
500 g	75	92	96	97	0.26
pH of solution					
pH 2	74	83	88	90	0.17
pH 7	44	71	80	90	0.13
pH 10	70	90	95	99.5	0.18
Temp. of solution					
10° C	40	66	78	86	0.15
80° C	71	90	94	97	0.20
Age					
100 g rat	77	94	98	99	0.22
400 g rat	35	66	74	89	0.11
Castration					
Controls	79	93	96	97	0.24
Castrates	50	63	80	87	0.14
Vit. C deficiency					
Controls	71	89	92	93	0.24
Deficient	89	97	98	98	0.34
Hyaluronidase					
Controls	65	89	90	92	0.27
Enzyme	93	99	100	100	0.45

Weight Application (Fig. 2)

It is to be expected that weight applied to the bulla would increase the rate of water loss in proportion to the weight applied.

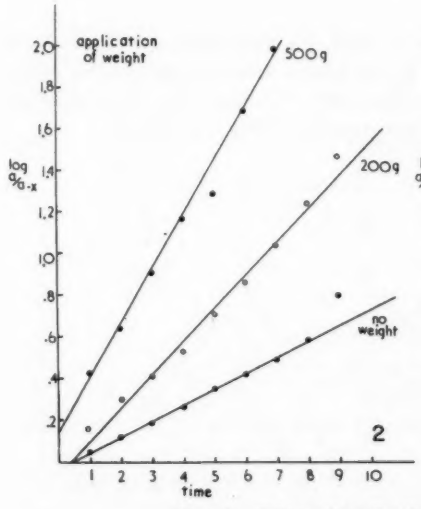
pH (Fig. 3)

McIlvaine–Lillie (31) citric acid – disodium phosphate buffers were used in the pH study. The figure shows the rate of water loss at pH 2, 10, and 7. The water retention was found to be greatest at pH 7 and to fall off on both the acid and alkaline sides.

TABLE II

Details of male castration experiment

	% water remaining at intervals of (min):									
	1	2	3	4	5	6	7	8	9	10
Controls										
Animal No. 1	40.5	16.6	9.0	6.5	4.5	2.8	2.0	1.5	1.5	1.2
No. 2	42.0	18.0	9.5	6.2	4.5	3.0	2.5	1.8	1.6	1.2
No. 3	49.2	26.0	17.2	12.8	9.5	7.6	5.2	3.8	3.0	2.0
No. 4	49.0	24.0	14.5	8.2	6.4	5.0	4.0	3.6	3.0	2.5
No. 5	46.2	23.0	12.4	8.0	7.5	5.5	4.2	3.8	3.6	3.2
Mean	45.4	21.3	12.5	8.3	6.5	4.8	3.6	2.9	2.5	2.0
Standard ± deviation	3.6	3.8	3.1	2.3	1.9	1.8	1.2	1.0	0.8	0.8
Castrate males										
Animal No. 1	77.0	59.5	46.0	37.0	31.5	28.0	24.5	19.8	17.2	14.6
No. 2	51.5	39.0	31.5	25.5	21.0	17.0	14.0	12.0	10.0	8.5
No. 3	61.0	43.0	32.5	27.0	22.2	19.1	15.4	14.0	11.6	7.6
No. 4	68.0	50.0	39.5	32.5	27.0	22.5	18.5	15.0	12.5	10.5
No. 5	76.0	61.0	50.0	41.0	34.5	29.5	24.5	21.0	16.5	14.5
Mean	69.0	51.2	39.2	32.0	26.8	23.6	20.0	16.9	14.4	11.1
Standard ± deviation	9.6	8.6	7.3	5.8	5.2	4.9	4.4	3.4	2.8	2.9



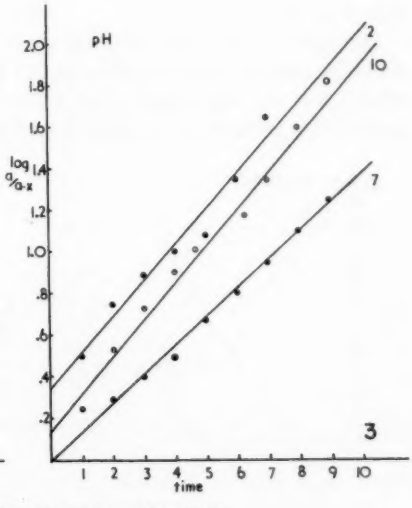
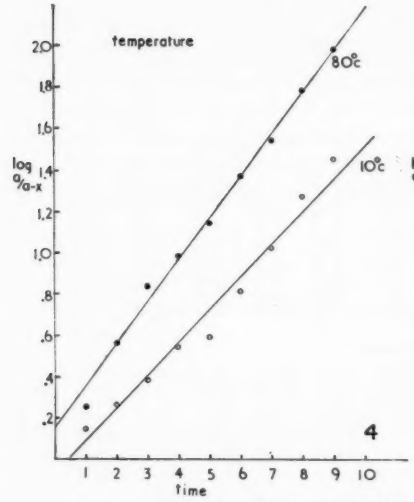


FIG. 2. Effect of application of weight on free bulla.
FIG. 3. Effect of variation of pH of injectant.

Temperature (Fig. 4)

Alterations in the temperature of the fluid injected caused wide variations in the rate of water loss, the hotter the injectant the more rapid the loss of fluid. The effects of solutions of normal saline at 10° C and 80° C are shown.



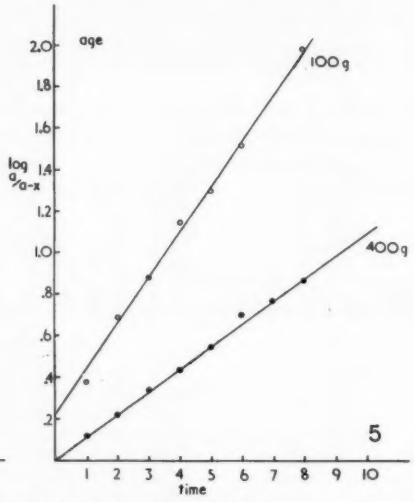
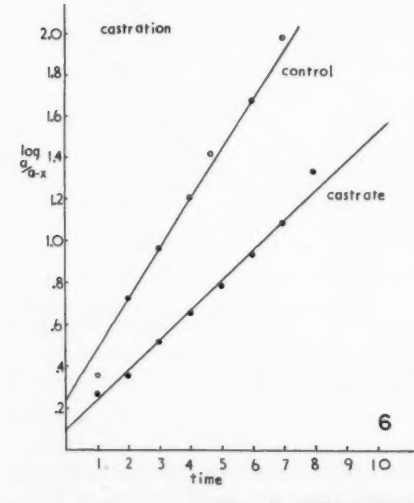


FIG. 4. Effect of variation of temperature of injectant.
FIG. 5. Effect of age of subject. Age expressed as weight of animal.

Age (Fig. 5)

The effect of age was obvious, even on injecting the solution. The bulla in the older animal felt firm and was dry to the touch, whereas that in the young animal was soft and rapidly lost water from the beginning. On the graphs the ages of the animals are expressed as their weights, 100 g and 400 g.



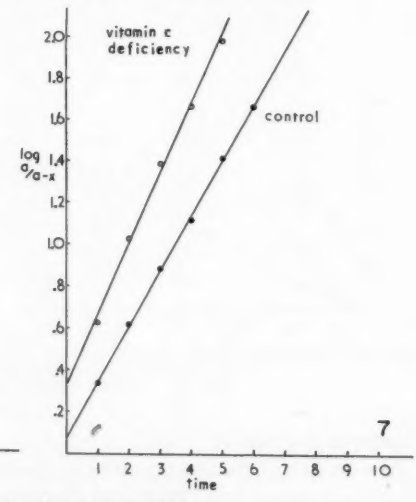


FIG. 6. Effect of castration of male subjects.
FIG. 7. Effect of vitamin C deficiency of subjects.

Castration, Male (Fig. 6)

The animals were castrated at 12 weeks, and the bullae were made 3 weeks postoperatively. It was found that the castrate lost water less rapidly than the control.

Vitamin C Deficiency (Fig. 7)

Adult male guinea pigs were maintained on the vitamin C deficient diet supplied by Nutritional Biochemicals Corporation. Control animals were maintained on standard guinea pig diet supplemented with green vegetables. The vitamin C deficient animals showed a greater rate of water loss from the bullae.

Hyaluronidase (Fig. 8)

These studies were performed with a solution of 150 t.r. units (Wyeth-Wydase) in 50 ml of normal saline. There was a quite definite excisable bulla formed, but, as would be expected, this rapidly gave up its water.

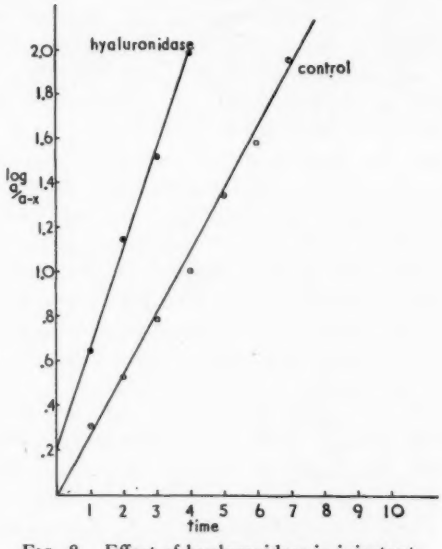


FIG. 8. Effect of hyaluronidase in injectant.

Comparisons should not be made between the various controls since these are not necessarily of the same age.

Discussion

When considering why the connective tissue should hold any of the injected water at all, it might at first seem reasonable to suppose that the water is trapped in the interstices of the fibrous tissue as in a sponge. This is further suggested by the experience that dense fibrous tissue, such as in the old animal,

holds the water more firmly than loose fibrous tissue, such as in the young animal. However, there are a number of points of experimental evidence which show that this is not the only cause. (*a*) Gross examination of the bullae shows the most fibrous ones to lose very little fluid over periods as long as an hour if not compressed. There is a pressure within the bulla set up by the force of the injection but the water is not extruded. When the top of the bulla is cut off, there is still only a minimal loss of water from its exposed surface, suggesting that the injected water is a part of the tissue of the bulla instead of being trapped in it as a separate phase. The bulla has the appearance of a jelly, and feels slimy but not wet, and even after being bisected horizontally, or even cut into many fragments, the pieces of the bulla continue to hold onto their water. (*b*) Histological examination of frozen sections through the bulla show that the network of fibrous tissue is very wide-meshed (Fig. 9) and that there is plenty of space between the fibers. Thus if the mesh were the only factor holding back the water, slicing through the bulla would liberate some, at least, of this trapped water. (*c*) Day's experiments (32, 33) showed that, after running water through the bulla, for several days it still retained its appearance, suggesting that the water was not held by a solution. He also showed that the rate of flow of fluid through a membrane could be altered by alternately removing and replacing material between the fibers. Thus, he suggested that the material between the fibers and not the fibers themselves controlled the flow. (*d*) If an irritant fluid is used to form the bulla (32, 28) there is an increase in the rate of flow. Similarly, when the bulla is made with hyaluronidase or when the pH or temperature is altered, as in the experiments conducted here, there is a rapid increase in fluid loss. With all these factors the fibers are seen grossly to be unchanged, but the rate of fluid loss is rapidly altered. Hvidberg (34) refers to the ability of connective tissue to hold in artificial states more water than it would normally, calling this the "swelling capacity" of connective tissue. It would seem therefore that the significant factor is not the mere presence of the fibers as a mechanical barrier, but some property between the fibers, i.e. the ground substance. Since the fluid started inside the bulla, and moved to the outside, the degree of resistance it encountered to its flow is the factor involved in determining the rate of flow, or velocity constant. If this resistance lies in the area of the ground substance, then it must be the cohesiveness of the ground substance that opposes the flow. This cohesiveness is in the bonding forces of the colloidal hydrophilic ground substance which hold together both the micellae of the ground substance and its contained water. Examination of the table of results shows that where there was a rapid loss of fluid the plateau *a* was higher, and where there was a slow loss of fluid the plateau was lower. Thus where the fluid loss was rapid it was also more complete, and as Kulonen (35) showed in dialysis experiments, increased water-binding capacity is related to a decreased water-binding strength. Where there is a denser ground substance, the constituent micellae are tightly packed together and hence the water of the ground substance is closely affected by

PLATE I

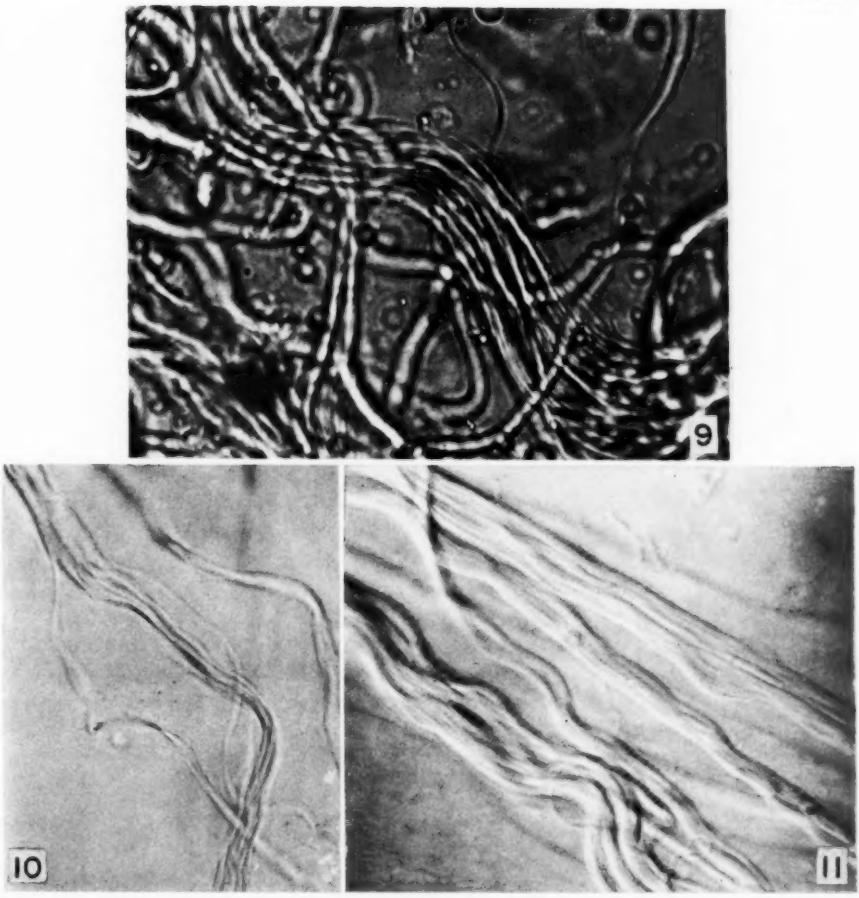


FIG. 9. The meshwork of collagenic fibers showing spaces between them. Frozen section through bulla. Oil immersion. × 250.

FIG. 10. Oil immersion appearance of frozen section through bulla made in normal 150-g male rat. × 300.

FIG. 11. Comparable appearance of normal 250-g rat. × 300.

them; where there is a loose ground substance, the relative volume of water to micellae is increased and hence the water is less affected by micellar charge. The introduction of water into this system alters further the water–micella balance. In the loose system where the water is already little influenced there will be a minimal hold on that newly introduced, and the water will be rapidly lost. In the dense system, although the micellae will become more widely separated by the introduction of water, their effect is still felt and the water is retained for a varying length of time. This has been the finding in the experiments where variations in the tissues were studied. In the age experiment, the younger tissue had a lesser hold on the injected water than the older tissue which is known to be more dense (36, 12, 37, 38, 39) (Figs. 10, 11). In the castration experiment the greater water retention was in the castrates' tissues, which are denser than the controls' tissues (40, 41) and are confirmed microscopically in this experiment. In the vitamin C deficiency study there was a lessened hold on the injected water by the deficient animals' tissues which have a less dense ground substance (12, 42, 43, 44). In the experiments where the injectant varied, the changes in the ability of the tissue to hold the injectant can be explained on the basis of alteration of charge of the ground substance micellae. The greatest viscosity of ground substance is at pH 6 (45) and a fall is found after adding alkali or acid (46). The electrostatic phenomenon of dye-binding by extracted mucopolysaccharides is pH dependent (19), and the internal bonds of collagen are greatly affected by alterations in pH (10). Electrometric titration of the dermis similarly shows a fall in binding power in the acidic range (11). There does not seem to be much published work on the effect of heat on the ground substance, but increase in energy from added heat causes a disruption of the internal bonds of collagen (10) and a similar effect is produced on mucopolysaccharides in vitro and in vivo by X-ray, ultraviolet, and infrared irradiation (47, 48). Hyaluronidase is a known depolymerizing agent, thus altering the state of charge of the ground substance. The findings of this experiment can therefore be correlated with known physicochemical properties, and it would seem that this method may be a useful tool in further examination of the ground substance.

Acknowledgments

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FURTHER STUDIES ON THE DUAL REQUIREMENT FOR PHENYLALANINE AND TYROSINE IN TISSUE CULTURE[1]

HELEN J. MORTON AND JOSEPH F. MORGAN

Abstract

Seventeen structurally related compounds were tested for their ability to substitute for phenylalanine or tyrosine in the nutrition of chick embryo heart fragments. DL-Alanyl-DL-phenylalanine replaced phenylalanine. All other compounds had negligible effects, and most were toxic at high concentrations. β-Phenylserine, a phenylalanine antagonist, actually prolonged the survival of chick heart cells but only if both phenylalanine and tyrosine were present. Similarly, optimal reversal of β-phenylserine toxicity was dependent on the presence of both amino acids. Although phenylalanine and tyrosine are not interconvertible in the present system, it has been shown that three phenylalanine antagonists, *p*-fluorophenylalanine, β-2-thienylalanine, and β-phenylserine, can be identified by their relationship to tyrosine, rather than to phenylalanine.

Introduction

Previous studies from this laboratory established that phenylalanine and tyrosine were not interconvertible by chick embryo heart fragments in vitro (1). This finding showed that the test system could be used to investigate each of the amino acids, in the presence of, but without interference from, the other. Accordingly, it was decided to test the ability of structurally related compounds to substitute for each amino acid in maintaining cell survival. Among the compounds tested was β-phenylserine, a known antagonist of phenylalanine in other systems (2). Under certain conditions this compound was found to increase cell survival, rather than to inhibit it. From these results it became apparent that tyrosine, while unable to substitute for phenylalanine, exerted a critical function in the reaction of the cells to certain phenylalanine antagonists.

Experimental

Chick embryo heart cultures were prepared and depleted of their intracellular reserves as described previously (1, 3). The freshly explanted fragments were maintained in balanced salt solution for 3 days. After this, cultures were divided into comparable groups which received fresh test media twice weekly until no living cells could be seen on microscopic observation. Details of the experimental design have been reported (3, 4, 5). All media employed were totally synthetic and, with the exception of the variations in tyrosine, phenylalanine, and related compounds under investigation, were identical with the formula of medium M150 (6, 7). The main variations in experimental media are described in Table I. The synthetic media were prepared from chemicals

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of the highest purity, including salts, amino acids, vitamins, and accessory growth factors. All test compounds were examined by paper chromatography and no contamination with either phenylalanine or tyrosine was detected.

Results

Ability of Structurally Related Compounds to Replace Phenylalanine

A synthetic medium was prepared which contained no phenylalanine but did contain tyrosine (M739, Table I). To this basal medium were added graded levels of each test compound, over the concentration range of 0.1 to 1000 mg per liter, and the effect on culture survival was determined. The results are summarized in Table II.

TABLE I

Amino acid composition of basic synthetic media used

Medium No.	Amino acid content
M150	Complete basal medium (7), entirely chemically defined; includes 50 mg/l. DL-phenylalanine, 40 mg/l. L-tyrosine
M665	L-Tyrosine omitted
M739	DL-Phenylalanine omitted
M1029	Both L-tyrosine and DL-phenylalanine omitted
M1318	M739 plus 10 mg/l. β-phenylserine
M2042	M1029 plus 10 mg/l. β-phenylserine

TABLE II

Ability of structurally related compounds to replace phenylalanine

Compound	Effect*	Active concn. (mg/l.)	Comment
L-Phenylalanine	++++	50.0	—
Phenylalamine	++	0.1	Toxic at 10 mg/l.
Dihydroxyphenylalanine	+	0.01	Toxic at 1 mg/l.
Shikimic acid	+	0.1	No effect at higher concn.
β-Phenylpyruvic acid	±	1.0– 10.0	Toxic at 100 mg/l.
β-Phenyllactic acid	+	0.1–100.0	Toxic at 1000 mg/l.
Acetylphenylalanine	+	10.0	No effect at higher concn.
Chloracetylphenylalanine	+	0.1–100.0	Toxic at 1000 mg/l.
DL-Alanyl-DL-phenylalanine	+++	1000.0	—
β-Phenylserine	−	—	Toxic at 1 mg/l.

*Cultures receiving full medium including phenylalanine (M150, Table I) survived an average of 35 days (++++). Cultures receiving the same medium lacking only phenylalanine (M739, Table I) survived an average of 15 days (−). Intermediate survivals are indicated by + signs.

It can be seen that none of the compounds tested completely replaced phenylalanine. Dihydroxyphenylalanine, shikimic acid, β-phenylpyruvic acid, β-phenyllactic acid, acetylphenylalanine, and chloracetylphenylalanine all showed a slight replacement activity. Phenylalamine (2-phenylethylamine) showed a moderate activity. Only DL-alanyl-DL-phenylalanine supported a prolongation of culture survival markedly beyond that of cultures in the M739 control medium. With the exception of shikimic acid and acetylphenylalanine, all compounds tested exerted a marked toxicity at high levels. Dihydroxyphenylalanine was unusual in exerting a toxicity at low concentrations.

Ability of Structurally Related Compounds to Replace Tyrosine

A basal medium was prepared which contained no tyrosine but did contain phenylalanine (M665, Table I). Various compounds related structurally to tyrosine were then added in graded levels over the concentration range of 0.1 to 1000 mg per liter and the effect on cell survival was determined. The results are presented in Table III.

TABLE III

Ability of structurally related compounds to replace tyrosine

Compound	Effect	Active concn. (mg/l.)	Comment
Tyrosine	++++*	40.0	—
Tyramine	−	—	Toxic at 100 mg/l.
3-Hydroxytyramine	−	—	Toxic at 10 mg/l.
3-Aminotyrosine	±*	0.1–1.0	Toxic at 10 mg/l.
Diiodotyrosine	−	—	No effect up to 100 mg/l.
Dihydroxyphenylalanine	−	—	Toxic at 10 mg/l.
β-Phenylpyruvic acid	−	—	Toxic at 1000 mg/l.
Shikimic acid	−	—	No effect up to 100 mg/l.
β-Phenylserine	−	—	Toxic at 100 mg/l.

*Cultures receiving medium including tyrosine (M150, Table I) survived an average of 35 days. Cultures receiving the same medium lacking tyrosine (M665, Table I) survived an average of 20 days; 35-day survival is indicated as ++++, 20-day survival is shown as −, ± indicates a questionable increase of 3 days.

It can be seen that tyramine, 3-hydroxytyramine, diiodotyrosine, dihydroxyphenylalanine, β-phenylpyruvic acid, and shikimic acid were completely ineffective in replacing the supplementary effect of tyrosine in the presence of phenylalanine. With the exception of shikimic acid and diiodotyrosine, all compounds tested exerted a marked toxicity at high levels, which was accompanied by a black, melanin-like deposit around the tissue.

Effect of β-Phenylserine on Culture Survival

The previous studies (1) had established that two phenylalanine antagonists, β-2-thienylalanine and *p*-fluorophenylalanine, exerted different effects which were dependent on the ratio of phenylalanine to tyrosine in the basal medium. Since β-phenylserine is known to be an antagonist of phenylalanine in other systems (2), its effect on tissue cultures was investigated. Graded levels of this analogue were added to complete medium M150, and to media deficient in either phenylalanine, or tyrosine, or both (Table I). The effects on culture survival were determined and are shown in Fig. 1.

In the absence of phenylalanine, the toxicity of β-phenylserine is marked, whether tyrosine is present (line A) or not (line C). It would appear, therefore, that tyrosine exerts no effect under these conditions. In the presence of phenylalanine, on the other hand, a marked stimulation from β-phenylserine is observed when tyrosine is present (line D), but this effect is not evident when tyrosine is absent (line B).

Reversal of β-Phenylserine Toxicity

Various combinations of phenylalanine and tyrosine were tested for their

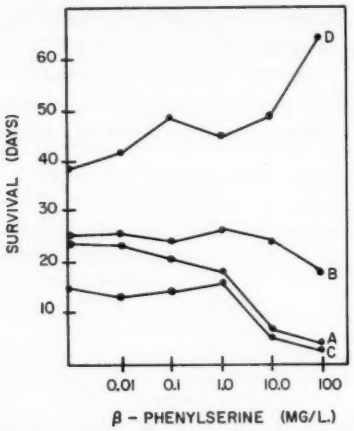

FIG. 1. Effect of graded levels of β-phenylserine added to medium lacking phenylalanine (curve A), lacking tyrosine (curve B), lacking both tyrosine and phenylalanine (curve C), or containing both tyrosine and phenylalanine (curve D).

ability to reverse the toxicity of β-phenylserine. The usual three media were prepared: M665, M739, M1029 (Table I), and to each was added a toxic concentration of β-phenylserine (10 mg per liter). Attempts were then made to reverse the toxicity by the addition of graded levels of phenylalanine or tyrosine. The results are shown in Fig. 2.

When tyrosine alone was added to toxic β-phenylserine no significant reversal was obtained (line A). When phenylalanine was added the maximum reversal

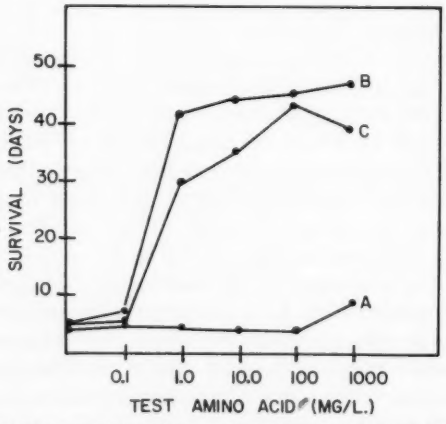

FIG. 2. Reversal of the toxicity of 10 mg/l. β-phenylserine: The effect of graded levels of L-tyrosine (curve A), graded levels of DL-phenylalanine in the presence of a constant amount of 40 mg/l. L-tyrosine (curve B), and graded levels of DL-phenylalanine in the absence of tyrosine (curve C).

was approximately equal regardless of whether tyrosine was present (line B) or absent (line C). However, the presence of tyrosine permitted essentially complete reversal at much lower concentrations of phenylalanine (compare lines B and C at 1.0 mg per liter).

Comparative Reactions of Three Phenylalanine Antagonists

Although amino acid antagonists have been used in many systems, the relationship between several antagonists and a single amino acid, in one system, has seldom been determined. It seemed of interest, therefore, to compare the effects of *p*-fluorophenylalanine and β-2-thienylalanine, as established previously in this system (1), with those obtained with β-phenylserine. The results are presented in Table IV.

TABLE IV

Comparative toxic and beneficial concentrations of three phenylalanine antagonists

Compound	Toxic* concn. (mg/l.)	PA/A ratio† for reversal	Beneficial‡ concn. (mg/l.)
p-Fluorophenylalanine	10.0	100:1	0.1
β-2-Thienylalanine	100.0	10:1	1.0
β-Phenylserine	10.0	10:1	100.0

*Basal medium contained tyrosine, but no phenylalanine.
†PA/A ratio is the ratio of concentration of phenylalanine to concentration of analogue required for complete reversal of analogue toxicity in the absence of tyrosine.
‡Basal medium contained both tyrosine and phenylalanine.

It can be seen that β-phenylserine exerted its toxicity on tissue cultures at the same concentration as did *p*-fluorophenylalanine (10.0 mg per liter). This concentration was 1/10th that required to demonstrate the toxicity of β-2-thienylalanine. The ratio of amino acid to antagonist required to reverse the toxicity was identical with β-phenylserine and β-2-thienylalanine (10:1) but was 10 times higher (100:1) with *p*-fluorophenylalanine; it was evident, therefore, that there was no consistent relationship between the toxic concentration of an antagonist and the amino acid:antagonist ratio required for the reversal of its toxicity.

The results shown in Table IV also indicate the concentration at which each antagonist caused an increase in cell survival when added to the complete medium containing both phenylalanine and tyrosine. β-Phenylserine was beneficial *only* under these conditions whereas the other compounds showed slight beneficial effects if either of the amino acids was present (1). The effect of β-phenylserine differed from *p*-fluorophenylalanine and β-2-thienylalanine in one other way. While the last two antagonists produced increases in survival at low levels followed by increasing toxicity (1), β-phenylserine was stimulatory at the highest concentration tested provided both amino acids were present. Moreover, this active concentration was 100 times the level which was toxic in the absence of phenylalanine (cf. Table II). Under these conditions, in the absence of tyrosine, phenylalanine alone would not even have reversed the toxicity (see Table IV, column 2, and Fig. 2, line C, 1.0 mg per liter).

Relationship between Tyrosine and the Phenylalanine Analogues

The unusual effect of tyrosine in the presence of β-phenylserine warranted further study of its relationship to phenylalanine analogues. These results are summarized in Table V. When tyrosine alone was added in the presence of a

TABLE V

Effect of tyrosine on the activity of three phenylalanine analogues

Analogue	Tyrosine alone	Tyrosine in the presence of phenylalanine
p-Fluorophenylalanine (1)	Increases the severity of the toxicity	No effect on reversal of toxicity by phenylalanine
β-2-Thienylalanine (1)	Causes slight reversal of toxicity	No effect on reversal of toxicity by phenylalanine
β-Phenylserine	No effect on development of toxicity	(Supplements the effect of phenylalanine) Converts a toxicity to a stimulation

toxic analogue a graded effect was seen. The toxicity of *p*-fluorophenylalanine was increased, that of β-phenylserine was unaffected, and there was a slight reversal of the β-2-thienylalanine effect. A totally different relationship was apparent when phenylalanine was also present. In this case, of the three antagonists, only β-phenylserine was affected. In the presence of both phenylalanine and tyrosine, the addition of β-phenylserine, rather than causing a toxicity, resulted in a marked increase in culture survival. The presence of tyrosine thus appeared to have converted the toxicity of β-phenylserine into a stimulation.

Discussion

Living cells in vitro are isolated from total-body reactions and are, therefore, an ideal tool for the study of metabolic side-reactions and alternate pathways. Since previous studies from this laboratory had shown that phenylalanine and tyrosine were not interconvertible by chick heart tissues (1), a survey of structurally related compounds was undertaken to seek reactions which might be involved in the metabolism of the two amino acids independent from each other.

It can be seen (Table II) that all of the compounds tested for their ability to replace phenylalanine showed some activity but only alanyl-DL-phenylalanine permitted good cell survival. This finding is in agreement with the results of Dickinson and Thompson (8), who found similar peptides active as phenylalanine substitutes in chick heart cultures. The slight activity of many other compounds used to replace phenylalanine suggests that non-enzymic mechanisms, similar to those found to be active in melanin formation from tyrosine and nitrophenols (9), might be responsible for their conversion to phenylalanine.

Eight compounds were tested for their ability to replace tyrosine but none showed any definite activity (Table III). Even tyramine was inactive, although the corresponding amine of phenylalanine showed moderate activity (Table II).

The derivatives and analogues tested for their ability to substitute for phenylalanine or tyrosine include at least one member of each established metabolic pathway for the two amino acids (10). The failure of these compounds to substitute completely suggests that none of the common pathways for the metabolism of these two amino acids are functional, and that the amino acids are used only for direct protein synthesis. However, if direct incorporation into protein were the only mechanism for utilization of phenylalanine and tyrosine, their uptake from the medium should show a direct relationship to the protein content of the tissue. In fact, the total protein of the tissue shows a progressive decrease (11), whereas the patterns of phenylalanine and tyrosine uptake remain fairly constant (12). These findings imply that there are other metabolic pathways active, for both amino acids, which do not involve any of the 11 inactive, or slightly active, compounds tested in the present survey.

Metabolic studies with the phenylalanine antagonist, β-phenylserine, were first made in 1909 (13). Subsequently, this compound has been used extensively in amino acid studies in animals (14, 15), in bacteria (16, 17, 18, 19), and as an antiviral agent in tissue culture (8). The present work has shown that this antagonist has a specific relationship to tyrosine. This is apparent from the data of Fig. 1, which show that phenylalanine obscures the toxicity of β-phenylserine until high levels of the latter are reached. However, the additional presence of tyrosine converts this high-level toxicity into a marked stimulation. There is, at present, no obvious explanation for this phenomenon. The failure of tyrosine to act against β-phenylserine, in the absence of phenylalanine, may be due to the fact that tyrosine alone will not support cell survival to any marked degree. Any beneficial effect would, therefore, be masked by death of the cells from phenylalanine deficiency. On the other hand, this stimulatory effect of β-phenylserine may need the presence of both phenylalanine and tyrosine. The latter explanation appears more probable since the curve for β-phenylserine (Fig. 1) in the presence of tyrosine, but in the absence of phenylalanine, shows a marked toxicity at higher levels (i.e. a lower survival than the no-phenylalanine control). These results are in contrast to those obtained with *E. coli* where tyrosine was found to enhance the toxicity of β-phenylserine (16).

A distinct, supplementary role for tyrosine in the presence of phenylalanine is also suggested by experiments on the reversal of a moderate β-phenylserine toxicity. It can be seen (Fig. 2) that tyrosine alone is ineffective, but that its presence, in addition to phenylalanine, reduces the amount of phenylalanine required for complete reversal to 1/100th of that necessary in its absence.

Studies in other systems suggest some possible reasons for the vital role of tyrosine under the present conditions. Liver and kidney slices oxidize both β-phenylserine and *p*-hydroxyphenylserine under certain conditions (14). Also, it has been postulated that dihydroxyphenylserine may be formed from tyrosine (20). If such reactions exist, and are reversible in the present system, active tyrosine metabolism may induce the formation of enzymes which also metabolize the hydroxyphenylserines and phenylserine itself. The presence of tyro-

sine would thus serve to reduce the active concentration of the antagonist. The formation of usable metabolites from phenylserine provides a plausible explanation for the resulting increase in cell survival.

One indication of the conversion of phenylserine to normal metabolites has been obtained by experiments with *E. coli* (21) in which the beneficial effect of phenylserine was attributed to its decomposition to serine, an essential amino acid for the strain in use. Specific involvement of serine seems unlikely in the present system since the basic medium contains a full supplement of amino acids (6, 7). Nor would this explanation clarify the critical role of tyrosine.

This activity of tyrosine indicates different functions for the three phenylalanine antagonists tested in the present, and previous (1), studies. The main distinguishing features are summarized in Tables IV and V. Various mutant strains of *E. coli* have shown differences in analogue response based on the function of tyrosine (22, 23). The present work has shown that such differences can also exist in an unselected cell population. This implies that the three antagonists may affect different pathways or different points on the same pathway. It is also apparent that these phenylalanine-antagonist-sensitive pathways differ most sharply, not in relation to phenylalanine, but in the manner and degree to which tyrosine is also involved. This conclusion is provocative, since tyrosine and phenylalanine are not interconvertible in the present system.

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MICROELECTRODE RECORDINGS FROM THE REGION OF THE NUCLEUS SOLITARIUS IN THE CAT[1]

R. S. SMITH AND J. W. PEARCE

Abstract

An extracellular microelectrode exploration of the region of the nucleus solitarius has been carried out in 30 adult cats with the purpose of studying central projections of cardiovascular afferent fibers. Of 375 discharging cells encountered, only 9 showed a cardiac periodicity which was not obviously artefactual. The latter cells were located in the region of, or ventral to, the caudal part of the nucleus solitarius. The infrequency with which such cardiovascular patterns of discharge were encountered leads to the conclusion that primary cardiovascular afferents do not provide the dominant influence on discharge of secondary neurones of the nucleus solitarius. It is suggested that interruption of other sources of afferent input to these cells might reveal the nature of transfer of baroreceptor discharge pattern across the first synapse of the visceral afferent pathways for cardiovascular receptors.

Introduction

The patterns of discharge of several types of cardiovascular afferent vagal neurones are well known (1). There are, however, no recordings of similar patterns of discharge from such afferent pathways beyond the first synapse, although good descriptions exist of cells in the region of the nucleus solitarius discharging with a respiratory rhythm (2, 3, 4, 5). Oberholzer (6) has used an ablation technique to reveal dependence of cardiovascular and respiratory reflexes on the integrity of the nucleus solitarius in the rabbit. His work suggests that cardiovascular afferents of the vagus synapse in the middle third of the extent of the nucleus. Bonvallet and Sigg (7) and Baumgarten *et al.* (8) have recorded activity in vagal rootlets, demonstrating at this level a degree of separation of primary visceral afferents according to functional modality. The recording of discharges with identifiable cardiac rhythm from cells of the nucleus solitarius would permit a better understanding of the degree of integration of visceral sensory impulses at the level of initiation of reflex responses.

Although reports of cardiovascular responses to punctate stimulation in the floor of the fourth ventricle are numerous (9), such stimulation may affect both adjacent cells and fiber tracts and hence does not yield precise information about the location of cardiovascular reflex centers. This is borne out by the lack of complete agreement in results obtained by stimulation (10, 11) and recording (2, 3, 5) methods as to the separation and extension of expiratory and inspiratory centers.

Successful recording from secondary neurones of cardiovascular afferents

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would also yield information about the transformation of discharge patterns across the synapse. Although afferents from the sensory cortex and spinal cord are known to end on cells of the nucleus solitarius (12, 13), and thus may be expected to modify the pattern of output of such cells, the existence of a cardiac rhythm of discharge in sympathetic efferents (14) suggests that such patterns must be preserved throughout some parts of the reflex pathways. A microelectrode exploration for spontaneous unit activity in the nucleus solitarius and adjacent regions of the medulla was therefore undertaken.

Methods

In 30 adult cats, anaesthetized either with sodium pentobarbital (35 mg/kg) or with chloralose (80 mg/kg), the floor of the fourth ventricle was widely exposed by removal of the occipital bone and most of the cerebellum. The pia mater was removed from a sufficient area to allow ready electrode insertion. In early experiments, to preserve normal intrathoracic negative pressure and the associated haemodynamic state required for continued activity of cardiovascular afferents, none of the usual precautions to reduce brain movement was adopted. In later experiments a bilateral thoracotomy and curarization with institution of artificial ventilation were employed to abolish respiratory movements of the brain stem. Needles were inserted subcutaneously in two extremities for recording the E.C.G., and a femoral vein was cannulated for the injection of drugs. The animal was then fixed in a stereotaxic machine provided with an electrode-advancing micrometer.

Rigidly mounted micropipettes, filled with Ringer–Locke solution (or with 5 *M* NaCl in the case of the smaller electrodes), varied in tip size between 1 and 20 μ. They were connected through a cathode follower to a conventional condenser-coupled amplifying system, the output of which was displayed on one beam of a cathode ray oscilloscope. The second beam was used to display the E.C.G. Photographic records were obtained using a moving-film camera which also registered 60 c.p.s. time-marking.

The locations of the electrode tracks were determined in most cases by measurements taken on the stereotaxic machine, and in some cases by histological examination. The method employed (15) consisted of *in situ* fixation of the brain with the electrode still in place, followed by removal of the electrode, serial sectioning of the brain at 50 or 100 μ using a freezing microtome, and subsequent examination of the sections mounted in glycerine. This method satisfactorily revealed the tractus solitarius and the electrode tracks.

Results

In the course of 121 microelectrode penetrations, a total of at least 375 unit discharges was recorded extracellularly. Some of the locations of the electrode tips are summarized in Fig. 1.

Initially, in order to sample from a larger number of cells believed likely to be discharging synchronously with the cardiac cycle, the larger-sized micro-

pipettes (12–20 μ) were used. Such electrodes yielded few single-unit recordings, but gave small amplitude multicellular recordings; no discharges with a clear resemblance to known cardiac afferent vagal activity were obtained. Considering the small size of the cells of the nucleus solitarius, and theorizing that a degree of asynchrony might obscure rhythms existing in masses of cells able to influence a larger electrode, it was decided to use a smaller size-range of micropipettes (less than 2 μ). While such electrodes usually recorded larger and single-unit potentials, cells were infrequently encountered and often damaged and spuriously discharged. Again no records were obtained of discharges with a close resemblance to those in primary cardiovascular vagal afferents.

With both sizes of microelectrodes a few discharges with a cardiac periodicity were encountered. In two cases an amplitude modulation strongly suggested that the discharge was spurious, produced indirectly by brain pulsation approximating a continuously discharging cell to the electrode tip. In nine other cases the discharge was not obviously artefactual and almost consistently began 75–85 milliseconds after the *R* wave of the E.C.G. and was of short duration (Fig. 2).

Discharges with a clear respiratory rhythm, not associated with amplitude modulation, were encountered 46 times; the discharges were almost always inspiratory in timing and were recorded from two areas, one superficial and one deep (Fig. 1). The remaining large majority of the discharges were continuous at intermediate frequencies, with or without superimposed random fluctuations in rate of discharge. Attempts to discover hidden cardiac rhythm, using frequency plots, almost universally failed to show a correspondence between given phases of the cardiac cycle and the fluctuations in frequency. The major types of discharge recorded are summarized in Table I, and some representative tracings are reproduced in Fig. 2.

TABLE I

Summary of discharges recorded

Discharge type	Number of cells encountered	General location	Remarks
Respiratory rhythm	46	Ventrolateral or dorsomedial	Usually inspiratory, rarely with superimposed cardiac rhythm
Continuous with or without erratic fluctuations of frequency	320	More commonly in nuclear regions	Wide frequency range, but never approaching that of peripheral baroreceptors
Cardiac rhythm	9	Near obex in region of nucleus solitarius	Single impulse or pair or short burst with each cycle, with first-impulse in almost all cases occurring 75–85 milliseconds after *R* wave of E.C.G.

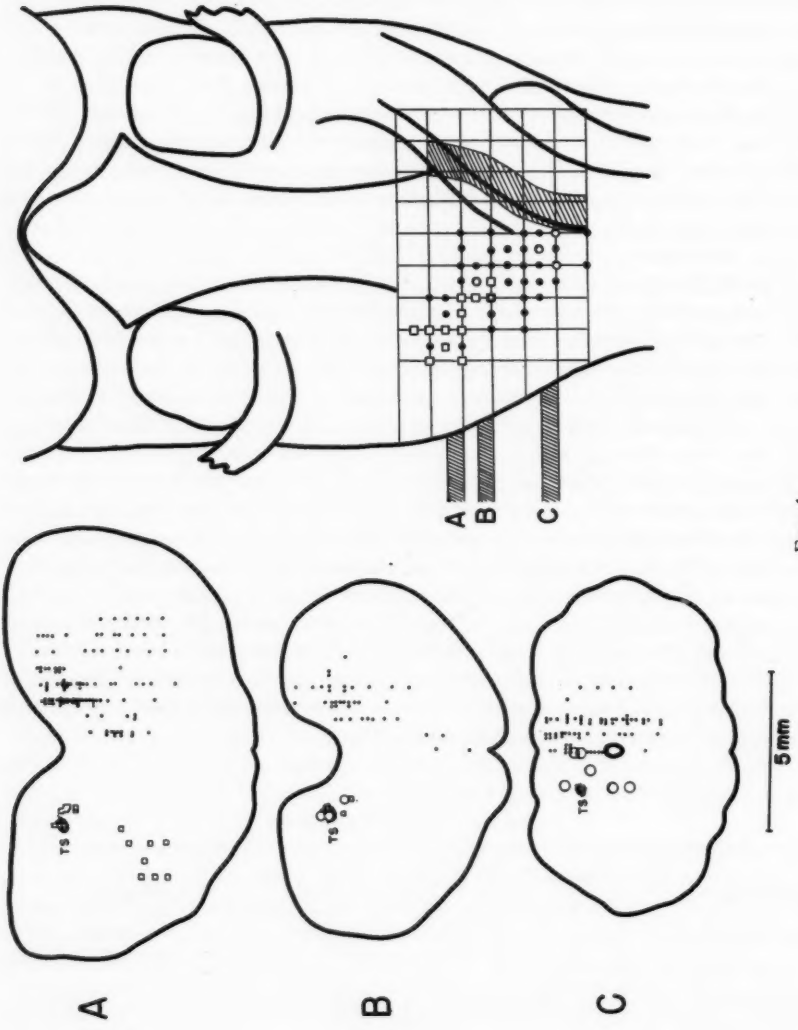

FIG. 1.

FIG. 1. The dorsal surface of the hindbrain is depicted at the *right*; on the millimeter grid are shown the locations of microelectrode penetrations which yielded cell discharges with unrecognizable pattern (filled circles) or, in addition to such discharges, one or more cell discharges with respiratory (open squares) or cardiac (open circles) rhythms. The points, although obtained on both sides of the midline, are represented on the left side only;

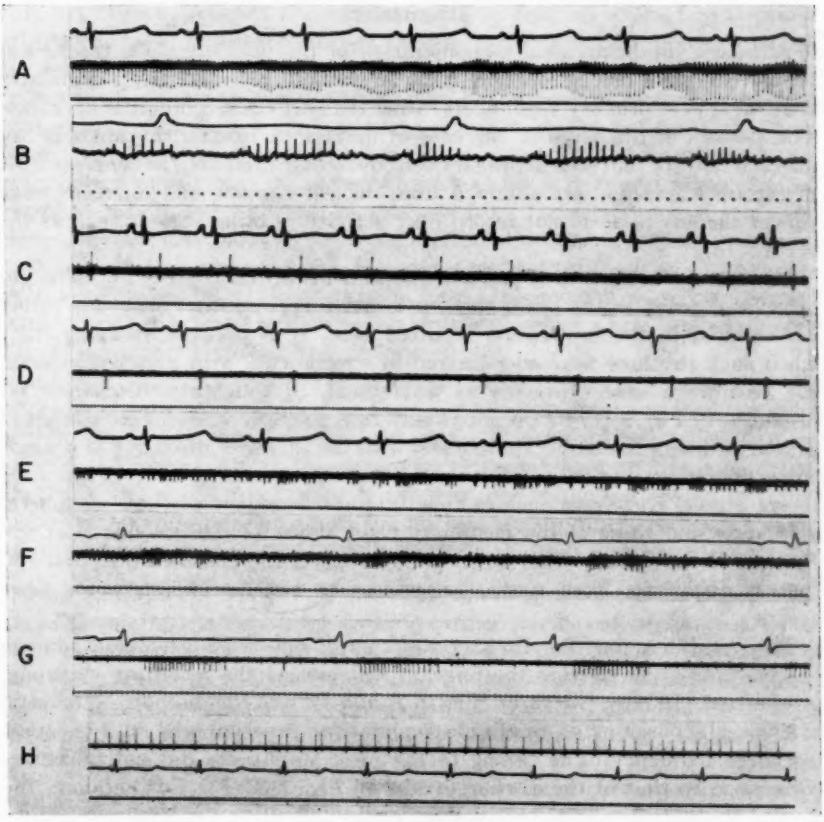

FIG. 2.

FIG. 1 (*concluded.*) the shaded area on the right of the midline depicts the relevant surface projection of the nucleus solitarius. The obex is 2 mm above the lowest horizontal grid line. A, B, and C correspond to the three sections, each 0.5 mm thick, shown in cross-sectional view on the *left* of the figure. In these selected planes, the types of discharge recorded from individual cells are presented to show also the depths at which the records were obtained. The two symbols on the left of the midline signify the discharge patterns described above; the locations of all cells encountered in each plane are shown as points on the right. TS, tractus solitarius. (Allowance has not been made for shrinkage of the fixed sections from which the outlines were drawn, whereas the recording points refer to stereotaxic co-ordinates obtained in the living preparation.)

FIG. 2. In each record are seen the E.C.G., the electroneurogram, and 60 c.p.s. time-marking. A and B show medullary cell discharges with an artefactual cardiac periodicity in amplitude but not in frequency. C–F show medullary cell discharges with a cardiac periodicity of frequency; the location of the cells is included in Fig. 1. G shows the discharge in a single aortic depressor *fiber*, presented for comparison with cell discharges above. H shows the only medullary cell discharge with a frequency modulation which could be shown, by frequency plotting, to be related to the cardiac cycle.

Discussion

Although the histological demonstration of the microelectrode tracks was too unrefined for precise location of the active units encountered, it is unlikely that these were primary vagal fibers rather than cells of second-order neurones. The pattern of discharge in the case of units with cardiac rhythm was not characteristic of that seen in primary cardiovascular afferents, the concentration of recorded activity was obtained from nuclear regions, and microelectrode tips of the size used do not record fiber activity in other regions such as the cortex (16).

In view of the absence of published reports of cardiac rhythm in discharges of cells recorded in the brain stem, it is not surprising that such was rarely encountered in the exploration reported here. It is possible, however, that when such rhythms were encountered in experiments with another purpose, the recordings were dismissed as artefactual. Although the discharges reproduced in Fig. 2 (C–F) do not closely resemble any known cardiovascular primary afferent discharge, comparison with the timing of discharge of a single aortic depressor fiber (Fig. 2, G), recorded at the conclusion of an experiment, shows a good correspondence in time of commencement of these peripheral discharges and those of the medullary cells, about 75 milliseconds after the *R* wave of the E.C.G. This observation supports the consideration that the central discharges were natural responses to primary baroreceptor fiber activity.

The possibility that the discharges of central cells were artefactual, due to vascular pulsation shifting the relevant cell toward the recording electrode, is admitted. In only two cases (Fig. 2, A and B) was the discharge obviously artefactual, consisting of amplitude modulations superimposed on a constant discharge frequency. The timing of the peak amplitudes did not, however, correspond to that of the discharges seen in Fig. 2 (C–F). Furthermore, the several fluctuations within each cardiac cycle, as well as a striking respiratory amplitude modulation, strongly suggest the venous pulse as the origin of the artefact. The discharges regarded as probably natural are related, on the other hand, to the beginning of the arterial pressure pulse but not to its peak. A final argument against the likelihood that all discharges with cardiovascular rhythm were artefactual is the infrequency with which they were encountered.

Although many penetrations of the region of the nucleus solitarius yielded recordings from a large number of cells (Fig. 1), only 2% of these cells showed a discharge with cardiac periodicity. Allowing that these few discharges were natural, it still remains to be explained that such were uncommon in view of the liberal input of afferent discharge with a striking cardiovascular rhythm. More sophisticated methods of analysis may reveal changes in excitability of cells of the nucleus solitarius reflected in subtle alterations in discharge frequency. A recognizable periodicity of such an alteration was only detected once (Fig. 2, H) by simple frequency plotting.

It has been noted that the nucleus solitarius receives afferent connections other than those in visceral nerves, and the latter probably represent one of several influences on the cells of the second-order neurones of the nucleus solitarius. Cells in reticular formation may be inhibited or excited by afferent connections. Although repetitive vagal stimulation has not been shown to modify the discharge of medial bulbar reticular units (17), the natural pattern of input of a variety of vagal afferents from both respiratory and cardiovascular receptors, associated with that of afferents from the cortex, spinal cord, and hypothalamus, may, however, give quite different results. Spinal interneurones with proprioceptive connections, the spontaneous activity of which resembles that encountered most frequently in our experiments, show considerable variability in functional properties but clearly represent a first level of integration of excitatory and inhibitory influences (18).

It is our contention that the rhythmic pattern of discharge of cardiovascular afferents is drastically modified at their first synapse, implying that the reflex activity they initiate will also depend on the state of systems other than the strictly cardiovascular. It would now seem a profitable approach to reduce the "mixing" which takes place at the synapses of the nucleus solitarius by extensive ablation of afferents to the nucleus and to other cell groups in the associated reflex loops.

NOTE ADDED IN PROOF: A personal communication from Dr. R. von Baumgarten reports recordings showing cardiac rhythm of discharge obtained with electrodes in the region of the nucleus tractus solitarius; the details of this work are to be published shortly in Pflüger's Archiv. Similar discharges have also been encountered by G. C. Salmoiraghli in a study of other brain stem neurones responding to cardiovascular maneuvers (to be published).

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ANTIBODIES TO ESTRONE-PROTEIN CONJUGATES

I. IMMUNOCHEMICAL STUDIES[1]

L. GOODFRIEND[2] AND A. H. SEHON

Abstract

The primary purpose of the present study was to determine if steroids, when conjugated by stable covalent bonds to proteins, could act as haptenic groups in inducing steroid-specific antibodies. Estrone was converted to estrone-17-isocyanate and the latter reacted with various proteins to give estrone-17-carbamido-protein conjugates. Rabbits were immunized with the conjugate prepared from human serum albumin and the resulting antisera were shown by various immunochemical techniques to contain antibodies specific to the estronyl residues of this conjugate. A turbidimetric assay method for the estimation of estrone-like compounds is described.

Introduction

In 1921 Geber (1) described the cyclical occurrence of urticaria in some women just prior to menstruation. Urbach (2) suggested that these symptoms were of an allergic nature since they could be elicited in the intermenstruum by the injection of the patient's own premenstrual serum and since in patients suffering shock following injection of estrone the premenstrual symptoms became manifest in subsequent menstrual cycles. In a study of a large series of patients with premenstrual symptoms, Zondek and Bromberg (3, 4) found that injection of estrone or estradiol benzoate into the patients resulted in allergic reactions (nausea, fever, urticaria, etc.). Intracutaneous testing with estrone, estradiol, progesterone, testosterone, deoxycorticosterone acetate, and other steroids gave the positive wheal and flare reactions characteristic of allergy. Healthy women showed no reaction. While the clinical findings of Zondek and Bromberg and of other workers (see review by Vallery-Radot (5)) suggested that steroids could act as allergens and that steroid-specific antibodies might be the etiological determinants of these premenstrual disorders, no conclusive proof has as yet been adduced for the presence of auto-antibodies to steroids in the sera of "premenstrual allergic" patients.

The purpose of the present study was to establish if steroids could indeed act as haptens, i.e., if antibodies specific to steroids could be elicited (in rabbits) by immunization with steroid-protein conjugates. If so, it was planned to develop sensitive and specific methods for the detection of such antibodies in the hope

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Contribution from the Departments of Biochemistry and Chemistry, McGill University, Montreal, Que. This work was supported by grants from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, and the National Research Council of Canada, Ottawa, Ontario. The results presented in this paper are taken from the Ph.D. thesis of L. Goodfriend, submitted in June, 1960, to the Department of Biochemistry, McGill University.
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that these methods might be employed for the detection of similar antibodies supposedly present in the sera of "premenstrual allergic" patients.

In 1941 Mooser and Grilichess (6) reported that androstenediol-protein conjugates failed to elicit rabbit antibodies to the steroid moiety of these conjugates. Recently, however, Erlanger, Lieberman, and their associates demonstrated that rabbit antibodies to testosterone, cortisone, progesterone, estrone, and deoxycorticosterone were formed by immunization with the corresponding steroid-protein conjugates (7, 8, 9, 10). Their results have been confirmed in the concurrent study to be reported here. In this investigation, rabbits were immunized with estrone-17-carbamido-HSA,* prepared according to a method previously described (11), and the resulting antisera were analyzed in cross-reaction and hapten-inhibition experiments for the presence of estrone-specific antibodies. A preliminary report of the results obtained was recently published (12).

Materials and Methods

1. Steroids and Other Chemicals

Estrone (m.p. 255° C) was kindly donated by Dr. G. Grant of Ayerst, McKenna and Harrison Co., Montreal, Que.; testosterone-17α-glucoside and 17α-hydroxycorticosterone-21-sodium succinate by Dr. W. C. Murphy of Ciba, Montreal, Que.; 16C^{14}-estrone was purchased from Charles E. Frosst and Co., Montreal, Que.; 17α-ethynyl-estradiol-17β from Sigma Chemicals, Chicago, Illinois; *n*-butyl- and α-naphthyl-isocyanates from Fischer Scientific Co., Montreal, Que. Estrone-17-isocyanate and 16C^{14}-estrone-17-isocyanate were prepared from 17-amino-estrone and 16C^{14}-17-amino-estrone (specific activity, 1350 c.p.m./mg),† respectively, according to the procedure previously described (11). Estrone-17-carbamido-glycine (EG) was prepared by reacting estrone-17-isocyanate with glycine (11).

2. Proteins

Rabbit serum albumin (RSA) was prepared from pooled rabbit serum by combined alcohol and ammonium sulphate precipitation methods. Chicken ovalbumin (OA, three times recrystallized) was prepared according to the method of Kekwick and Cannan (13). Bovine serum albumin (BSA, three times recrystallized) was purchased from Armour and Co., N.Y., and human serum albumin (HSA, pooled) from Connaught Medical Research Laboratories, Toronto.

3. Conjugates

(*a*) The method previously described (11) was used to couple estrone-17-isocyanate to RSA, BSA, and HSA to form the corresponding estrone-17-

*NOTE: HSA = human serum albumin. BSA = bovine serum albumin. BGG = bovine gamma globulin. RSA = rabbit serum albumin.

†Solutions of this and all other substances analyzed for radioactivity were prepared in 0.1 *N* NaOH and counted as "infinitely thin" plates in a windowless Q gas counter. Counts were made in duplicate over time intervals required to give less than 5% error.

carbamido-protein conjugates: C17-E-RSA, C17-E-BSA, and C17-E-HSA. Similarly $16C^{14}$-labelled C17-E-BSA (specific activity, 120 c.p.m./mg) and C17-E-OA (specific activity, 85 c.p.m./mg) were prepared by coupling $16C^{14}$-estrone-17-isocyanate to BSA and OA, respectively. The *n*-butyl- and α-naphthyl-isocyanates were coupled to BSA and HSA. The conjugates were purified by precipitation with ammonium sulphate (11) and by acetone extraction: 4 volumes of acetone at −10° C were added slowly with continuous stirring to 1 volume of a 1% sodium bicarbonate solution of the conjugate (2 g%) maintained at 0–5° C. As acetone extraction rendered the OA conjugate "insoluble", the latter was further purified by prolonged dialysis against dilute alkali (pH 9.5) until a constant specific activity was obtained. Control proteins consisted of the corresponding albumins, subjected to the same coupling and purification conditions employed for the preparation of the conjugates.

Table I shows the number of residues coupled to the different proteins as determined by ultraviolet spectrophotometric and (or) ninhydrin colorimetric methods (11). The number of estronyl residues obtained by these methods for $16C^{14}$-C17-E-BSA, i.e. 21 estronyl residues per molecule of BSA, was in good agreement with the number of 23 determined by radioactive counting.* Using the latter method, 11 estronyl residues were found for $16C^{14}$-C17-E-OA.

TABLE I

Number of residues and electrophoretic mobilities

Protein or conjugate	Number of free-amino groups*	Number of residues*	Number of residues†	Electrophoretic mobility,‡ $-\mu\times10^5$
Untreated RSA	50	—	—	3.84
Control RSA	49	—	—	3.88
C17-E-RSA	24	25	26	5.80
Untreated BSA	49	—	—	3.94
Control BSA	49	—	—	4.18
C17-E-BSA	24	25	27	6.52
$16C^{14}$-C17-E-BSA	27	22	19	6.06
n-Butyl-BSA	15	34	—	8.18
α-Naphthyl-BSA	11	38	—	8.44
Control HSA	49	—	—	4.31
C17-E-HSA	24	25	27	6.63
n-Butyl-HSA	18	31	—	—
α-Naphthyl-HSA	16	33	—	—

*Determined by ninhydrin method (11).
†Determined by ultraviolet spectrophotometric method (11).
‡In 0.1 *M* phosphate buffer, pH 6.0 (11).

In 0.1 *M* phosphate buffer at pH 6.0, the conjugates and control proteins displayed single peaks in the Tiselius electrophoresis apparatus (Spinco Model H). The conjugates were found to have significantly higher anodic mobilities

*The number of estronyl residues was calculated using the formula $(c/s)\times(M/272)$ where c and s denote the specific activity of conjugate and of $16C^{14}$-17-amino-estrone, employed as standard, respectively, M the molecular weight of the conjugate, and 272 the molecular weight of the amine.

than those of the corresponding control proteins (Table I). Some of the conjugates and control proteins dissolved in veronal buffer (ionic strength 0.1, pH 8.6) were analyzed in the Model E Spinco optical ultracentrifuge at 59,780 r.p.m. and at $20.0 \pm 0.1°$ C. The control proteins gave rise to one sedimenting peak essentially identical with that obtained for the untreated proteins, indicating that no serious alterations in protein structure had resulted under the coupling and purification conditions employed to prepare the conjugates. Similarly, a single peak was obtained in the case of *n*-butyl-BSA, but the estronyl- and naphthyl-derivatives of BSA displayed two peaks (Fig. 1).

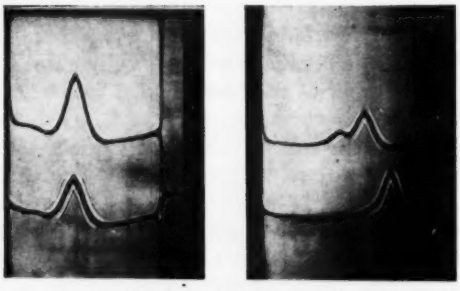

FIG. 1. Schlieren patterns obtained in the ultracentrifuge for proteins and conjugates.
Left: Photograph taken 95 minutes after full rotor speed was attained. Upper curve represents control BSA and lower curve, unreacted BSA.
Right: Photograph taken 54 minutes after full rotor speed was attained. Upper curve represents C17-E-BSA and lower curve, control BSA.

(*b*) Estrone-17-O-carboxymethyloxime-BSA (C17-E-O-carb.-BSA) was kindly supplied by Dr. F. Erlanger, Columbia University, N.Y. Arsanilic-azo-HSA (AS-HSA) was prepared by Mr. A. Froese in this laboratory.

4. Rabbit Antisera

(*a*) Antisera to C17-E-HSA were obtained from rabbits injected intravenously (via the marginal ear vein) with 1 ml of a 2% sterile saline solution of C17-E-HSA, three times a week for 4 weeks. Blood samples were collected periodically starting 6 days after the last injections and analyzed by ring test (14) with the immunizing antigen. Fifty milliliters of blood was taken from each rabbit whose serum gave a positive ring test and 2 days later another 50 ml was taken. Some portions of the antisera were stored at 5° C without preservative. Other aliquots, in 5-ml vials, were frozen and stored at $-20°$ C. The latter procedure ensured uniformity of properties of the sera over prolonged periods of storage, which was felt particularly necessary for reproducibility of results in turbidimetric experiments. The antisera were not pooled.

(*b*) Antisera to HSA and BSA were obtained from stock antisera prepared in this laboratory and antiserum to arsanilic-azo-BGG (AS-BGG) was provided by Mr. A. Froese.

5. Precipitin Reaction (15)

All solutions were prepared in borate buffer (0.15 *M*, pH 8.0). To a series of round-bottom test tubes, each containing 1.0 ml of antiserum (or diluted antiserum), were added 1.0-ml volumes of antigen solution in varying concentration. The antigen–antibody mixtures were incubated for 2 hours at 37° C followed by 48 hours at 5° C. The contents were then centrifuged for ½ hour at 5° C. The supernatants were decanted and the precipitates were washed with 2.0-ml volumes of ice-cold saline. The process of centrifugation and washing was repeated three times in all. The washed precipitates were analyzed* for nitrogen content by the method of McKenzie and Wallace (16), employing a Markham-type micro-Kjeldahl apparatus. The antibody content of the antiserum was calculated by multiplying the maximum nitrogen value, corrected for antigen nitrogen added, by the standard factor of 6.25.

6. Hemagglutination

The bis-diazotized-benzidine (BDB) method of Pressman *et al.* (17) was employed as adapted in this laboratory for the detection of antibodies in allergic sera (18). To couple antigen to rabbit erythrocytes ("sensitization"), 3 ml of antigen solution (1.0 mg/ml), prepared in phosphate buffer (0.1 *M*, pH 7.3), was mixed with 0.1 ml of a 50% suspension of washed erythrocytes, and 0.5 ml of BDB – phosphate buffer (1:15) was added to the suspension. The reaction was allowed to proceed at room temperature for 15 minutes. The mixture was then centrifuged, the cells were washed with 3.5 ml of diluent† and resuspended in 2.5 ml of diluent. Portions of 0.05 ml of the sensitized cell suspension were dispensed in 0.5-ml volumes of halving serial dilutions of antiserum contained in test tubes. The tubes were allowed to stand overnight at room temperature to permit the development of specific hemagglutination patterns. The titer was taken as the inverse of the highest dilution of antiserum which still gave a positive hemagglutination pattern. For each experiment, two controls were used: (*a*) in one series of tubes the antiserum was replaced by the diluent; (*b*) in another series non-sensitized cells were used.

The cross reactivity of different antigens was determined by inhibition experiments. For this purpose 0.1 ml of solutions of the test antigens was added to the serial dilutions of the antiserum prior to the addition of the sensitized cells and the reduction in the hemagglutination titer was ascertained.

7. In Vitro Anaphylaxis

Young female guinea pigs were passively sensitized by intraperitoneal injections of rabbit antisera. After 48 hours as recommended by Coulson (19) for maximum sensitization, the animals were killed by a sharp blow on the head, the uterine horns were excised and mounted in the muscle bath of a Schultz–Dale apparatus. The muscle bath, of 15-ml capacity, was immersed in a water

*Thanks are due to Mr. A. Froese for performing these analyses.

†Obtained by a 100-fold dilution with phosphate buffer of complement-inactivated serum from the rabbit supplying the erythrocytes.

bath thermostatically maintained at 37.5° C. A two-way stopcock arrangement permitted entry and evacuation of Tyrode solution (20) from the muscle bath. The uterine segments were mounted by fixing one end of the tissue to an oxygen inlet and the other to a lever pen mechanism. Contractions were recorded in ink on a kymograph rotating at 5 mm per minute.

The muscle segments were allowed to come to a steady base line. Subsequently, 1.0-ml volumes of test-antigen solutions were added to the Tyrode medium bathing the tissue. All solutions were prepared in Tyrode; the concentration of antigen employed (1.5 mg/ml) was such as to completely desensitize the muscle to this antigen after a single challenge.

For hapten inhibition, a segment of one uterine horn was challenged with antigen and served as a control, while the homologous segment of the other horn of the same uterus was incubated with test inhibitor in Tyrode solution for 5 minutes and then washed with Tyrode solution prior to challenge with antigen. In experiments performed in the absence of inhibitors, contraction invariably occurred in 1 minute or less. However, to establish the occurrence of inhibition after incubation with a test hapten, a period of at least 5 minutes was allowed after addition of the protein antigen. To test the possibility that the uteri may have become refractory, histamine was generally added to the bath after challenge with antigen.

8. Turbidimetry

The course of the antigen–antibody reaction was followed turbidimetrically in a Beckman DU spectrophotometer. Measurements of the light (at a wave length of 436 mμ) scattered at 90° were made on the "percentage of transmission" scale at fixed intervals of time. A tungsten lamp served as the light source, in conjunction with the blue filter provided with the Beckman fluorescent attachment. All solutions were prepared in buffered saline. The cell was filled with 3.0 ml of buffered saline and the reading of the light scattered at 90° was registered; this served as the cell blank. To the 3.0 ml of buffered-saline solution, 0.2 ml antiserum was added, the cuvette was inverted three times, and a reading was taken. This reading, minus the cell blank, corresponded to the light scattered by the antiserum alone (antiserum blank.) In the uninhibited reactions, 1.0 ml of buffered saline and 2.0 ml of antigen solution were pipetted into the cuvette and a reading was made: this reading, minus the cell blank, served as the antigen blank. To the 3.0 ml of antigen solution 0.20 ml of antiserum was added, the cuvette was inverted three times, and was placed in the spectrophotometer. Readings were made every minute for the first 10 minutes after addition of the antiserum and every 2 minutes thereafter. These readings were corrected by subtracting the cell, antigen, and antiserum blanks.

In hapten-inhibition experiments, 1.0 ml of test hapten prepared in buffered saline was used in place of the 1.0 ml of saline ordinarily employed. The percentage of reduction of turbidity due to a particular test-inhibitor was calcu-

lated on the basis of the turbidity values at 20-minutes obtained in the presence and in the absence of the inhibitor.

The experiments were conducted at room temperature (about 25° C).

Experimental Results

1. Cross Reactions

By ring test, all the estrone-protein conjugates reacted with the antisera to C17-E-HSA. On the other hand, except for the immunizing carrier protein, HSA, none of the control proteins gave a reaction. In addition the *n*-butyl and α-naphthyl conjugates of BSA failed to react. Similar results were obtained with the quantitative precipitin and hemagglutination techniques as shown in Tables II and III, respectively. It is apparent from these results that the antisera formed to C17-E-HSA contained at least two types of antibodies: antibodies to HSA and antibodies specific to the estrone-protein conjugates.

TABLE II

Cross reactions of antigens with rabbit antiserum (9-3) to C17-E-HSA by quantitative precipitin reaction

	Antigen added (μg/ml serum*)								
	0.5	1	2	4	8	16	32	64	128
Antigen	Total nitrogen precipitated (μg/ml serum*)								
C17-E-HSA	13	19	31	49	89	142	151	50	18
Control HSA			19	20	19	20	27		
16C^{14}-C17-E-BSA			24	31	52	93	94		
n-Butyl-BSA	No reaction								

*Serum diluted 1:3 with borate buffer (0.15 *M*, pH 8.0).

TABLE III

Cross reactions of antigens with rabbit antiserum (9-2) to C17-E-HSA by the BDB-hemagglutination method

	Titer	
	Cells sensitized with:	
Free antigen added*	C17-E-HSA	Control HSA
Uninhibited reaction	102,400	6,400
C17-E-HSA	0	0
Control HSA	51,200	0
n-Butyl-HSA	51,200	0
C17-E-BSA	3,200	6,400
Control BSA	102,400	6,400
n-Butyl-BSA	102,400	6,400
α-Naphthyl-BSA	102,400	6,400
C17-E-RSA	6,400	6,400
Control RSA	102,400	6,400
16C^{14}-C17-E-OA	6,400	6,400
Control OA	102,400	6,400

*The amount of each antigen added per tube was 300 μg.

This was confirmed in experiments using the in vitro anaphylaxis technique (Figs. 2 and 3).* Except for HSA, none of the control proteins elicited contraction of uteri sensitized with antisera to C17-E-HSA. Following desensitization to HSA, challenge with the estrone-protein conjugates, i.e. with either estrone-17-O-carboxymethyloxime-BSA or C17-E-BSA invariably elicited contractions. Moreover, desensitization to one antigen prevented contraction with the other and vice versa. The uteri still responded to HSA following desensitization to either of the two estrone-BSA conjugates. On the other hand, prior desensitization to C17-E-HSA, the immunizing antigen, abolished the response to both HSA and the various conjugates.

The failure of the control preparations of BSA, RSA, and OA to react with the antisera to C17-E-HSA demonstrated the heterologous nature of these proteins in relation to HSA, the carrier protein of the immunizing conjugates. It was concluded, therefore, that antibodies reacting with all the estrone-protein conjugates were specifically directed to a common antigenic determinant which did not reside in the protein moieties of these conjugates. The identification of this common antigenic determinant with the estronyl residue was substantiated by the results obtained in hapten-inhibition experiments using the in vitro anaphylaxis and turbidimetric techniques.

2. Hapten Inhibition: In Vitro Anaphylaxis

Typical results obtained with guinea pigs passively sensitized with antisera to C17-E-HSA are shown in Fig. 4. In the uninhibited cases the uterine tissues contracted to C17-E-BSA and to control HSA. Prior incubation of uterine tissues from the same animals with 100 or 200 μg of estrone-17-carbamido-glycine completely inhibited contraction to C17-E-BSA but was without effect on the response to HSA.

The effect of prior incubation of sensitized uteri† with 25, 50, and 100 μg of estrone-17-carbamido-glycine is shown in Fig. 5. Complete inhibition of contraction to heterologous conjugates was obtained.

In some experiments, the horns of the sensitized uterus were segmented into base and tip halves. The tip half of one horn was challenged with antigen, while the tip half of the other horn was incubated with test hapten prior to challenge with antigen. The same procedure was followed for the base halves. Twenty micrograms each of estrone,‡ 17α-ethynyl-estradiol-17β, and estrone-17-carbamido-glycine were equally effective§ in preventing the response to C17-E-BSA (Fig. 6).

The specificity of the inhibition shown by the estrone derivatives was tested in a number of ways. As much as 500 μg of testosterone-17α-glucoside or

*In this and all other figures relating to anaphylaxis experiments, the essential parts of the results obtained have been juxtaposed by omitting parts of the tracing during which the tissue was washed or allowed to relax.

†In these experiments the animals were sensitized with 1.0 ml volume of antiserum diluted 1:4 with saline.

‡Ten milliliters of a solution of estrone in Tyrode (ca. 2.0 μg/ml) was added to the bath.

§One-milliliter volumes of antiserum diluted 1:4 with saline were used for sensitization.

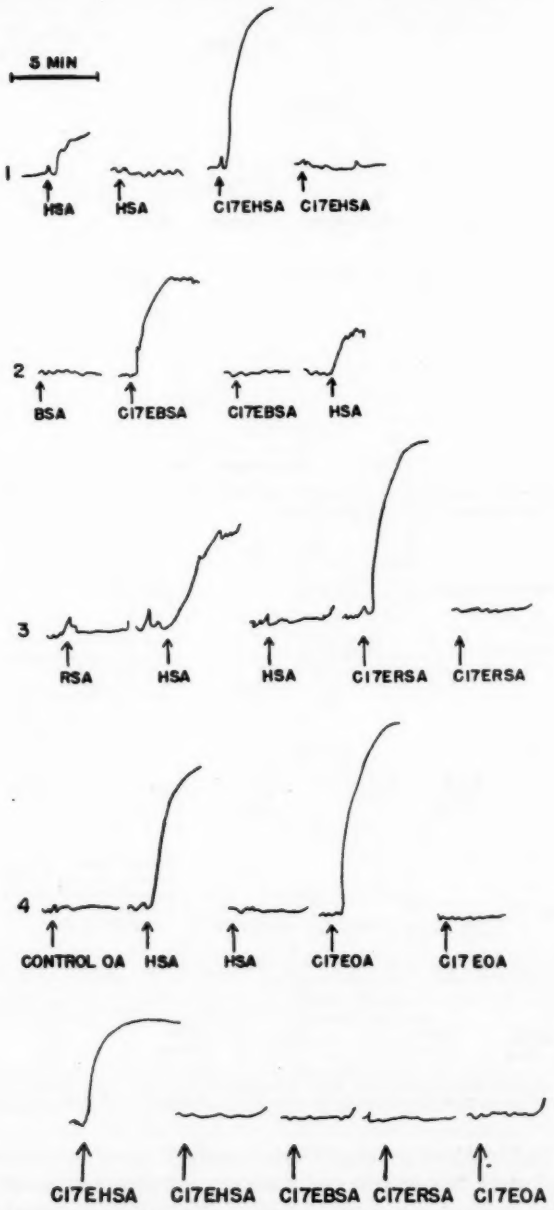

FIG. 2. In vitro anaphylaxis of guinea pig uteri passively sensitized with antiserum to C17-E-HSA on challenge with test antigens.

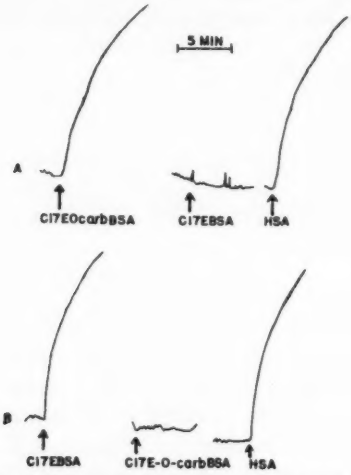

FIG. 3. In vitro anaphylaxis of guinea pig uteri passively sensitized with antiserum to C17-E-HSA on challenge with test antigens.

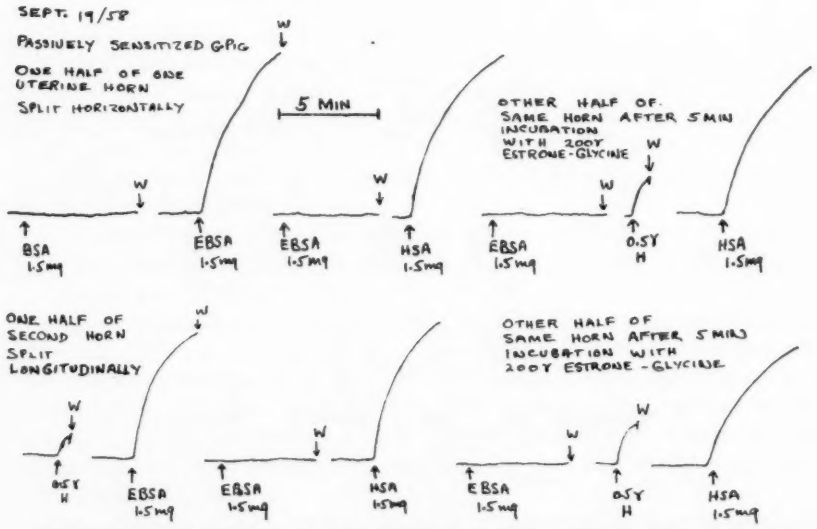

FIG. 4. Hapten inhibition of in vitro anaphylaxis. Guinea pig uterus passively sensitized with antiserum to C17-E-HSA. H represents histamine and W designates wash.

1000 μg of 17α-hydroxycorticosterone-21-sodium succinate failed to inhibit contraction of uteri from guinea pigs passively sensitized with rabbit antiserum to C17-E-HSA when challenged with C17-E-BSA (Fig. 7). On the other hand, in control experiments with appropriate segments of the uterine horns of the

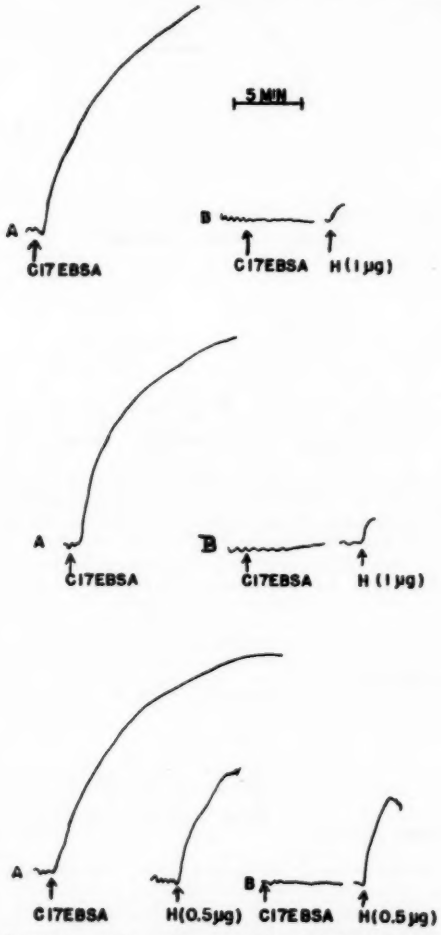

FIG. 5. Hapten inhibition of in vitro anaphylaxis. Three guinea pig uteri passively sensitized with antiserum to C17-E-HSA. A represents uninhibited reaction of one horn. B represents other horn of same uterus previously incubated with 25 μg (top), 50 μg (middle), and 100 μg (bottom) of estrone-17-carbamido-glycine.

same guinea pigs, 200 μg of estrone-17-carbamido-glycine was effective in inhibiting contraction to C17-E-BSA (Fig. 7). To exclude the possibility of nonspecific inhibition by estrone and the C17-estrone derivatives, a number of unrelated antigen–antibody systems were tested for inhibition with these compounds. As indicated in Fig. 4, estrone-17-carbamido-glycine was ineffective in preventing contraction of anti-C17-E-HSA-sensitized uteri on challenge with control HSA. Similarly none of the estrone derivatives had any effect on

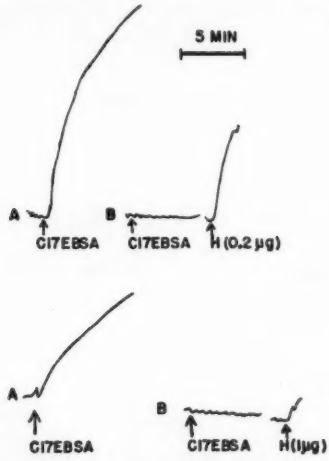

FIG. 6. Hapten inhibition of in vitro anaphylaxis. Guinea pig uterus passively sensitized with antiserum (1:4) to C17-E-HSA. Top: one uterine horn. Bottom: other horn. A represents uninhibited reaction, B the reaction after previous incubation of uterine segment with 20 μg each of 17α-ethynyl-estradiol-17β (top) and estrone (bottom).

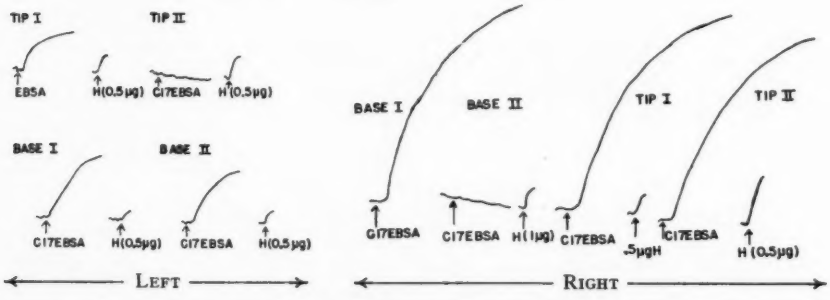

FIG. 7. Specificity of hapten inhibition of in vitro anaphylaxis. Guinea pig uterus passively sensitized with antiserum to C17-E-HSA.
Left: tip I and base I: uninhibited; tip II and base II: test-inhibited with 200 μg of estrone-glycine and 500 μg of testosterone-glucoside, respectively.
Right: base I and tip I: uninhibited; base II and tip II: test-inhibited with 200 μg of estrone-glycine and 1000 μg of 17α-hydroxy-corticosterone-21-sodium succinate, respectively.

anaphylaxis of uteri separately sensitized with antisera to BSA, HSA, AS-BGG when challenged with BSA, HSA, and AS-HSA, respectively (Fig. 8).

3. Hapten Inhibition: Turbidimetry

Table IV gives the turbidity values (represented by the spectrophotometer readings) at different times for various antigen concentrations and with a constant amount (0.20 ml) of antiserum for the C17-E-BSA – anti-C17-E-HSA

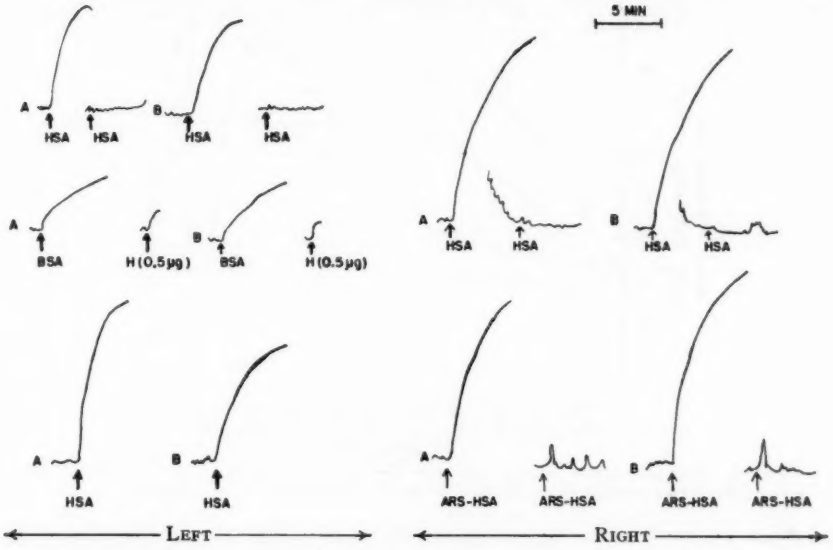

FIG. 8. Specificity of hapten inhibition of in vitro anaphylaxis.

Left: Three guinea pig uteri separately sensitized with antiserum to HSA (top and bottom) and BSA (middle). A, one horn of each uterus; uninhibited. B, other horn of same uterus test-inhibited with 200 μg of 17α-ethynyl-estradiol-17β (top), 20 μg of estrone (middle), and 200 μg of estrone-17-carbamido-glycine (bottom).

Right: Two guinea pig uteri separately sensitized with antiserum to HSA (top) and AS-BGG (bottom). A, one horn of each uterus; uninhibited. B, other horn of same uterus test-inhibited with 200 μg or estrone-17-carbamido-glycine.

TABLE IV

Turbidity values* registered as percentage of transmission versus time for C17-E-BSA – anti-C17-E-HSA reaction†

Time (minutes)	Concentration of C17-EBSA (μg N/ml)					
	4.0	8.0	10	16	32	64
1	6.2	7.3	7.5	8.0	4.9	5.6
2	7.6	9.2	9.9	10.5	5.3	5.7
3	8.5	11.0	11.7	11.7	6.1	5.8
4	9.3	12.4	13.0	12.6	6.7	5.9
5	10.2	13.5	13.8	13.3	7.0	6.0
6	10.8	14.5	14.5	14.0	7.3	6.1
7	11.6	15.1	15.1	14.4	7.5	6.2
8	12.1	15.7	15.4	14.5	7.7	6.4
9	12.6	16.1	15.7	14.6	7.8	6.5
10	12.9	16.3	15.9	14.6	7.9	6.6
12	13.2	16.4	16.3	14.6	8.1	6.7
14	13.3	16.5	16.6	14.6	8.3	6.8
16	13.4	16.5	16.8	14.6	8.5	6.8
18	13.5	16.5	17.0	14.6	8.6	6.8
20	13.5	16.5	17.0	14.6	8.6	6.8

*Corrected for cell, antigen, and antiserum blanks.

†Antiserum to C17-E-HSA (6-2) was employed in this and subsequent turbidimetric experiments.

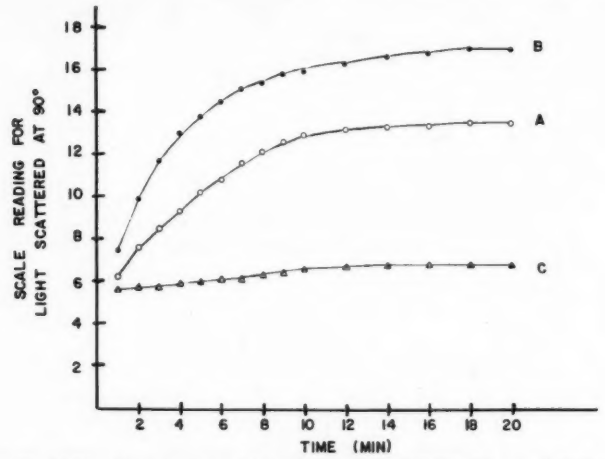

FIG. 9. Turbidity–time curves obtained for the C17-E-BSA – anti-C17-E-HSA reaction. Antigen concentrations: A, 4 μg N/ml; B, 10 μg N/ml; C, 64 μg N/ml.

system. Figure 9 shows typical curves obtained on plotting these values. As can be seen, maximum turbidity values were approached after about 20 minutes. Similar curves were obtained for the BSA – anti-BSA system, which was used as a control.

Precipitin-like curves (Fig. 10) were obtained by plotting the turbidity values at 20 minutes against the corresponding antigen concentration. From these

TABLE V

Inhibition of turbidity* due to C17-E-BSA – anti-C17-HSA reaction†

Time (minutes)	Uninhibited reaction	Inhibitor				
		Estrone (2 μg/ml)	17α-Ethynyl-estradiol-17β		Estrone-17-carbamido-glycine	
			4 μg/ml	8 μg/ml	5 μg/ml	10 μg/ml
1	7.5	2.4	1.4	1.5	1.0	0.2
2	9.9	3.4	1.6	1.7	1.2	0.2
3	11.7	4.0	1.9	1.9	1.4	0.2
4	13.0	4.4	2.1	2.1	1.5	0.2
5	13.8	4.6	2.4	2.3	1.6	0.2
6	14.5	4.8	2.6	2.4	1.8	0.2
7	15.1	5.0	2.7	2.5	1.9	0.2
8	15.4	5.0	2.9	2.6	1.9	0.2
9	15.7	5.1	3.0	2.7	2.0	0.2
10	15.9	5.2	3.2	2.8	2.1	0.2
12	16.3	5.4	3.5	2.9	2.2	0.2
14	16.6	5.6	3.7	3.0	2.3	0.2
16	16.8	5.7	3.9	3.1	2.4	0.2
18	16.9	5.7	4.0	3.2	2.5	0.2
20	16.9	5.8	4.0	3.2	2.6	0.2

*The turbidity values were registered as percentage of transmission and were corrected for cell, antigen, and antiserum blanks.
†Antigen concentration, 10 μg N/ ml.

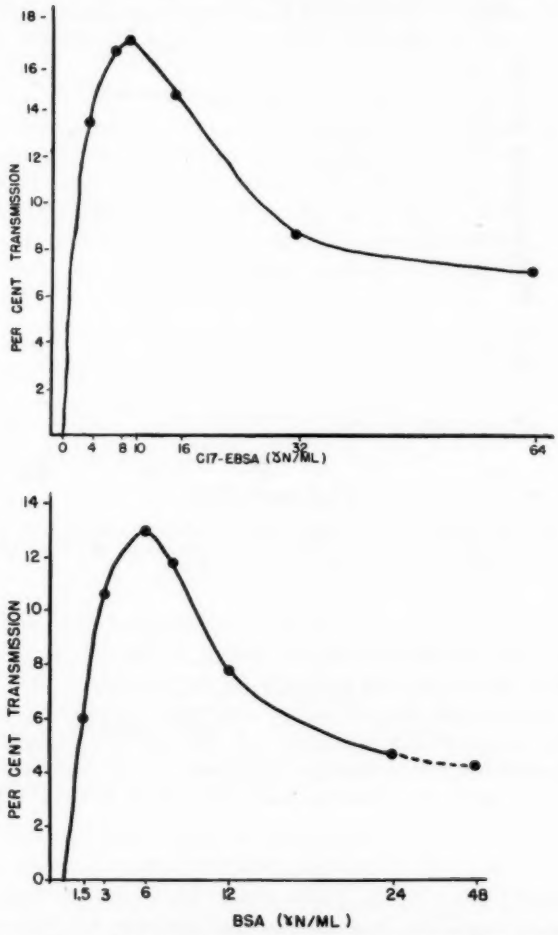

FIG. 10. Top: Plot of the 20-minute turbidity due to C17-E-BSA – anti-C17-E-HSA reaction against corresponding antigen concentration.
Bottom: Plot of the 20-minute turbidity due to BSA – anti-BSA reaction against corresponding antigen concentration.

curves the optimum antigen concentrations giving maximum turbidity were determined for the two antigen–antibody systems. For the amounts of antisera employed (0.2 ml) the optimum antigen concentrations were found to be 10 μg N/ml of C17-E-BSA and 6 μg N/ml of BSA, respectively. These antigen concentrations were subsequently employed in hapten-inhibition studies.

Table V gives the turbidity–time values obtained for the reaction between C17-E-BSA and antiserum to C17-E-HSA in the absence and in the presence

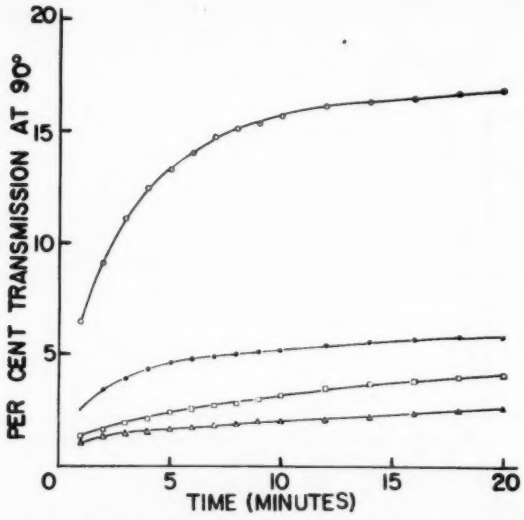

FIG. 11. Hapten inhibition of turbidity due to C17-E-BSA – anti-C17-HSA reaction. Curves drawn from values given in Table V. ○ uninhibited, ● estrone (2 μg), △ 17α-ethynyl-estradiol-17β (4 μg), and □ estrone-glycine (5 μg).

of various amounts of estrone, 17α-ethynyl-estradiol-17β, and estrone-17-carbamido-glycine. Typical curves are shown in Fig. 11. It is evident that these steroids caused a marked reduction in the amount of light scattered at 20 minutes; the corresponding reduction expressed in percentages are given in Table VI. To establish the specificity of this inhibition, testosterone-17α-glucoside, 17α-hydroxycorticosterone-21-sodium succinate, the 2,6- and 3,4-dimethylphenols, and (*d*,*l*)-tyrosine were also employed as test inhibitors (Table VI).

TABLE VI

Inhibition of turbidity due to C17-E-BSA – anti-C17-E-HSA reaction

Test inhibitor	Amount added (μmoles)	% inhibition*
Estrone	0.007	66
Estrone-17-carbamido-glycine	0.013	85
Estrone-17-carbamido-glycine	0.026	99
17α-Ethynyl-estradiol-17β	0.013	76
17α-Ethynyl-estradiol-17β	0.026	89
17α-Hydroxy-corticosterone-21-sodium succinate	0.44	31
Testosterone-17α-glucoside	0.44	76
2,6-Dimethyl-phenol	1.6	0
3,4-Dimethyl-phenol	1.6	16
(*d*,*l*)-Tyrosine	1.1	0

*Calculated on the basis of the turbidity readings at the 20-minute mark for the uninhibited and inhibited reactions.

All substances which were found to inhibit the C17-E-BSA – anti-C17-E-HSA reaction were tested also in the BSA – anti-BSA system: no inhibition could be detected.*

The inhibition of the C17-E-BSA – anti-C17-E-HSA reaction by the C17-estrone derivatives suggested that the turbidimetric technique might be employed to assay such derivatives. This possibility was tested by subjecting the C17-E-BSA – anti-C17-E-HSA reaction to inhibition with 1, 2, 4, and 8 μg of 17α-ethynyl-estradiol-17β. A plot of the percentage inhibition at the 20-minute mark against the corresponding inhibitor concentration is shown in Fig. 12. This curve was employed to assay a solution of 17α-ethynyl-estradiol-17β of "unknown" concentration prepared by a colleague in this laboratory.

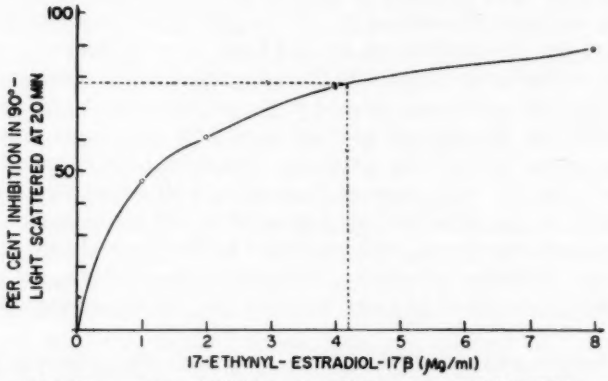

FIG. 12. Plot of percentage inhibition of turbidity due to C17-E-BSA – anti-C17-E-HSA reaction obtained for various concentrations of 17α-ethynyl-estradiol-17β. The broken line represents the percentage of inhibition obtained with a solution of 17α-ethynyl-estradiol-17β of concentration ca. 4.0 μg/ml.

From the percentage inhibition obtained with the "unknown" solution, 4.2 μg/ml was calculated as the concentration of this steroid solution (Fig. 12). The solution prepared contained 4.0 μg/ml, which demonstrates the feasibility of assaying solutions of steroids by inhibition of specific antigen–antibody aggregation.

Discussion

The results of the cross-reaction experiments demonstrated a twofold specificity for the antibodies produced to C17-E-HSA: (i) antibodies directed to the determinants of the protein moiety (HSA) of the immunizing antigen; (ii) antibodies directed to a determinant shared in common by all the C17-estrone-protein conjugates, which was, therefore, considered to be the C17-estronyl residue.†

*Essentially identical results were obtained for the HSA – anti-HSA system.

†This does not exclude the possibility that a heterogeneous spectrum of antibodies (21) was formed against different portions of this residue.

The identification of the estronyl residue as the common antigenic determinant reacting with antibodies formed to C17-E-HSA was established in hapten-inhibition experiments. Thus, incubation of sensitized uteri with small amounts of estrone and of the C17-derivatives of estrone completely prevented anaphylaxis on addition of C17-E-BSA. The high estrone-specificity of these antibodies was demonstrated by the inability of the testosterone and deoxycorticosterone derivatives (in relatively large amounts) to inhibit anaphylaxis to C17-E-BSA. The specificity of the inhibitory capacity of the C17-estrone derivatives was further demonstrated by their inability to inhibit the heterologous systems: HSA – anti-E-HSA, HSA – anti-HSA, BSA – anti-BSA, and AS-HSA – anti-AS-BGG.

Similarly, the light scattered at 90° due to C17-E-BSA – anti-C17-E-HSA interaction was markedly reduced by estrone and C17-estrone derivatives, while tyrosine and the dimethylphenols showed little or no inhibitory activity. The appreciable inhibition obtained with the testosterone and corticosterone derivatives indicates that antibodies formed to the estrone-protein conjugates, while highly specific to the estronyl residues, were able to accommodate to some extent the general steroid ring structure. Assuming little or no effect due to differences in the C17 side-chains of these steroids, the relatively lower inhibitory capacity of the corticosterone compared to the testosterone derivatives may be interpreted as due to steric hindrance by the 11α-hydroxyl group at the antibody site. A similar reduction in inhibitory capacity due to the presence of an 11α-hydroxyl was found by Lieberman *et al.* (10) for various steroid–antibody systems.

Since *n*-butyl-carbamido-BSA did not react with the antiserum to C17-E-HSA in the in vitro anaphylaxis, hemagglutination, and quantitative precipitin reactions, it may be concluded that no specific antibodies were formed to the carbamido linkage as such. This was further supported by the lack of cross reaction of α-naphthyl-carbamido-BSA with the antiserum in the hemagglutination experiments. It should be stressed, however, that these results do not rule out the possibility that, in the reactions obtained with the estronyl-carbamido-proteins, the linkage was an integral part of the haptenic group.

The demonstration of haptenic activity for steroids adds credence to the postulate of Zondek and Bromberg (3, 4) that auto-antibodies to steroid hormones may be responsible for premenstrual disorders of an allergic nature. The present studies cannot be regarded as an experimental model duplicating processes which may occur in man and which may result in the induction of steroid-allergic states. Rather, they suggest the possibility that antibodies with specificities directed to endogenous steroids may be produced. A closer experimental analogue for the "natural" formation of such antibodies might be to immunize animals with conjugates prepared with isologous proteins. In point of fact, such a procedure was adopted early in the present studies. Rabbits were immunized with the C17-estrone conjugate of rabbit serum albumin; the resulting antisera, while weak, appeared to contain precipitating

antibodies specific to the estronyl residues. However, these experiments were of a decidedly preliminary nature and further investigations along these lines are required.

The concept of auto-antibodies to steroids as etiological determinants in premenstrual disorders has by no means been universally accepted, and other mechanisms for the development of the observed symptoms have been suggested (22). Obviously, of great significance for the clarification of the etiology of these disorders would be the actual detection of steroid-specific antibodies in suspect allergic sera. It is apparent from the findings of Zondek and Bromberg (3, 4) and of other investigators (5) that the steroid-specific antibodies, if present at all, could be found in the circulation of the patients particularly at the height of the disorders, i.e., just prior to menstruation, since sensitivity could be transferred at this time to the skin of normal patients by passive transfer of the "allergic" sera. The preparation of steroid-protein conjugates such as those of the present studies and of Erlanger *et al.* (7, 8) makes possible the detection of such antibodies by the highly sensitive BDB-hemagglutination technique (18). Some attempts were made in this direction with human sera obtained from six women showing "premenstrual allergic" symptoms,* using erythrocytes sensitized with the estrone-HSA conjugate. However, the results obtained were either negative or were considered not sufficiently definite owing to the extremely low titers (1:10–1:40) which were obtained. It is noteworthy, though, that the titers obtained were consistently higher for the premenstrual than for the midmenstrual sera. Further experiments along these lines, with more human sera, are required to establish the etiology of "premenstrual allergy" on a firm basis.

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ANTIBODIES TO ESTRONE-PROTEIN CONJUGATES

II. ENDOCRINOLOGICAL STUDIES[1]

L. GOODFRIEND[2] AND A. H. SEHON

Abstract

It was demonstrated that (i) antiserum to estrone-17-carbamido-HSA neutralized the 6-hour uterotropic activity of exogenous estrone in immature rats, and (ii) the estrone-17-carbamido-proteins were devoid of estrogenic activity at the 5.0-mg dosage level in immature female rats.

The preparation of estrone-protein conjugates and of antibodies specific to the estronyl residues, as demonstrated in the preceding paper (1), made it possible to investigate problems of endocrinological interest. In the present paper are reported the results of preliminary studies on the antihormonal activity of antisera to estrone-protein conjugates and on the hormonal activity of estrone-protein conjugates.

A. Inhibition of Estrogenic Activity

It was shown (1, 2) that rabbit antiserum formed to estrone-17-carbamido-HSA* (C17-E-HSA) contained antibodies which combined in vitro with estrone or various derivatives of estrone. It was of interest to determine if this antiserum could inhibit the in vivo activity of the estrogenic steroid hormones, since it is conceivable that such antiserum might be of therapeutic value in counteracting pathological conditions in man which are dependent upon stimulation by estrogens, e.g. in the case of estrogen-dependent tumors (3). In principle such antibodies could be induced by injection of estrone conjugates prepared with human serum proteins. In the present study, the neutralizing effect of antiserum to C17-E-HSA on the uterotropic activity of exogenous estrone in the immature rat was examined.

MATERIALS AND METHODS

(i) Antisera

The rabbit antiserum to C17-E-HSA employed in these experiments contained approximately 1.2 mg of antibody per ml.† Rabbit antiserum to HSA was used as a control; its antibody content was not determined, however.

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Contribution from the Departments of Biochemistry, Experimental Medicine and Chemistry, McGill University, Montreal, Que. This work was supported by grants from the National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Maryland, and the National Research Council of Canada, Ottawa, Canada. The results presented in this paper are taken from the Ph.D. thesis of L. Goodfriend, submitted in June, 1960, to the Department of Biochemistry, McGill University, Montreal, Que.

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*NOTE: HSA = human serum albumin.

†This value was estimated from the maximum antibody nitrogen precipitated by C17-E-BSA (1).

(ii) Assay of Estrogenicity

The method of assay was based on the 6-hour uterotropic effect of estrogens in the immature female rat (4, 5). The rats employed, weighing between 35 and 55 grams, were selected from 21- to 24-day-old lots of an inbred ("hooded") strain bred at the Royal Victoria Hospital, Montreal. All solutions were prepared in buffered saline* and administered intraperitoneally in 2-ml aliquots. The animals were kept without food and water for 6 hours after injection; they were then sacrificed by an overdose of chloroform and weighed to the nearest tenth of a gram. The uteri were excised by stripping away the connective and vaginal cervical tissue and cutting at the utero–tubular junctions. They were blotted lightly in a uniform manner and immediately weighed in closed, tared containers to the nearest tenth of a milligram. The uterine to body (U/B) ratio was determined for each rat by dividing the weight of the uterus in milligrams by the body weight in grams. The increase in the U/B ratio obtained with a test solution above that obtained with saline was taken as a measure of the estrogenicity of the test solution and was compared with that obtained with 0.2 μg of estrone.

Experiments and Results

In preliminary experiments it was established that a minimum of 0.2 μg of estrone was required to obtain an appreciable increase (about 50%) in U/B ratios compared with those obtained with buffered saline (cf. also (5)). Experiments were, therefore, designed to determine the capacity of the antiserum to C17-E-HSA to inhibit the estrogenic activity of this level of estrone. This was diluted with buffered saline and with a buffered-saline solution of estrone so that a 2-ml aliquot of the final antiserum solution contained 0.2 μg of estrone and 0.80 ml of antiserum. The antiserum to HSA, which was employed in control experiments, was similarly treated.

Rats were divided into four groups: one group was injected with 2-ml aliquots of buffered saline; the second with 2-ml aliquots of buffered saline containing 0.2 μg of estrone; the third with 2-ml aliquots of the control solution containing estrone and antiserum to HSA; the fourth with 2-ml aliquots of the solution containing estrone and antiserum to C17-E-HSA. The animals were assayed 6 hours after injection. The U/B ratios obtained for each group are shown in Table I.

It may be seen from this table that the estrone – anti-HSA solution gave an average U/B ratio equal to that obtained with estrone alone. On the other hand, the estrone – anti-C17-E-HSA solution led to an appreciably smaller average U/B ratio than that obtained with estrone alone or with the estrone – control serum solution. The difference in the average U/B ratios obtained with antiserum to C17-E-HSA and antiserum to HSA was found to be significant by Student's statistics (6) ($P < 0.1\%$).

*Buffered saline consisted of a solution made up of equal volumes of 0.15 *M* NaCl and 0.15 *M* phosphate buffer, pH 7.3.

TABLE I

Inhibition of exogenous estrone in immature rats

Rat No.	Uterine to body ratios obtained with:* Buffered saline	0.2 μg estrone	0.2 μg estrone +0.8 ml antiserum to HSA	0.2 μg estrone +0.8 ml antiserum to C17-E-HSA
1	0.83	1.38	1.27	0.89
2	0.72	0.82	1.24	1.21
3	0.72	0.96	1.27	0.92
4	0.84	0.98	1.23	0.91
5	0.80	1.29	1.13	0.82
6	0.86	0.81	0.88	0.92
7	0.75	0.96	0.93	0.70
8	0.77	1.31	1.06	0.76
9	0.71	1.10	0.96	0.68
10	0.77	0.94	1.04	0.87
11	0.73	1.16	0.93	0.71
12	0.78	1.14	1.29	0.69
13	0.79	1.14	0.93	0.87
14	0.70	0.93	1.07	0.80
15	0.72	1.00	0.98	0.68
16			1.04	0.80
17			0.98	1.06
18			1.19	0.96
19			0.94	1.04
20			0.95	0.72
21			1.02	0.68
22				0.91
23				0.97
24				0.85
Average U/B†	0.76	1.06	1.05	0.85
Standard deviation	±0.051	±0.174	±0.126	±0.122

NOTE: The "pooled" standard deviation for the U/B values listed in the last two columns is ±0.124, which leads to a value of $P \ll 0.001$ in terms of Student's t statistics ($t = 5.4$).

*All solutions were made up in buffered saline and administered intraperitoneally in 2-ml volumes.

†U represents the uterine weight in milligrams; B represents the body weight in grams.

DISCUSSION

Hooker and Boyd (7) have calculated that drug-specific antiserum could be expected to neutralize the pharmacological activity of drugs requiring only relatively few molecules for therapeutic action. In the present study, the capacity of 0.8 ml of anti-C17-E-HSA serum to neutralize the uterotropic activity in rats of 0.2 μg of estrone was examined. The amount of antiserum employed contained 1.2 mg or 0.75×10^{-8} moles of antibody (taking the molecular weight of antibody as 160,000). Assuming two combining sites per antibody molecule, the number of moles of antibody sites available for combination with estrone was therefore 1.5×10^{-8}. Comparing this number with the number of moles of estrone equivalent to 0.2 μg, i.e., 0.7×10^{-9}, this means that about 20 combining sites were available for combination with each molecule of estrone. Apparently this ratio of antibody sites to estrone resulted in the formation of inactive estrone–antibody complexes to an extent sufficient to suppress the uterotropic activity of the hormone in the immature rat. On the other hand, Lieberman

and associates (8) reported that injections of 1-ml aliquots of antiserum to C17-E-O-carb.-BSA per day for a period of 6 days did not protect spayed female rats against a total of 15 μg of estrone in aqueous suspension administered over the same period of time. It is possible that in their experiments the antiserum used was not sufficiently potent to neutralize the rather large amount of estrone administered. However, it is worth noting that their experiments with antitestosterone serum indicated that this antiserum inhibited the androgenic activity of hormonally active testosterone preparations (8).

B. Hormonal Activity of Estrone-Protein Conjugates

Experiments were made to determine if the C17-estrone-protein conjugates possessed estrogenic activity. For this purpose, assays were made of the C14-labelled conjugate with bovine serum albumin, a conjugate which contained on the average 21 C17-estronyl residues per protein molecule. For comparison, assays were made of 17-amino-estrone and estrone-17-carbamido-glycine prepared previously (1). The 6-hour uterotropic assay described in Part A of this paper was employed.

Experimental Results and Discussion

Table II shows the U/B ratios obtained with (i) saline, (ii) 0.2 μg of estrone, (iii) 40 μg each of 17-amino-estrone and estrone-17-carbamido-glycine, and (iv) 5.0 mg of 16-C^{14}-C17-E-BSA. It can be seen that, whereas 40 μg of the C17-amino or glycine–ureido derivatives of estrone gave average U/B ratios comparable to that of 0.2 μg of estrone, the C17-estrone-protein conjugate did not increase the U/B values above the basal level obtained with saline.

TABLE II

6-Hour uterotropic activity of 17-amino-estrone, estrone-17-carbamido-glycine, and 16C^{14}-C17-E-BSA

	Uterine to body ratios obtained with:				
	Saline	0.2 μg estrone	40 μg 17-amino-estrone	40 μg estrone-17-carbamido-glycine	5.0 mg 16C^{14}-C17-E-BSA
Expt. I	0.71	1.00	1.07	0.83	0.77
	0.82	1.05	1.38	1.31	0.71
	0.70	1.15	1.15	1.00	0.69
Expt. II	0.76	1.19	1.22	1.05	0.80
	0.70	1.08	1.39	0.90	0.73
	0.75	1.06	0.96	0.70	0.71
Average	0.73	1.09	1.19	1.13	0.74

Note: U represents uterine weight in milligrams; B represents body weight in grams. All solutions were made up in buffered saline and administered intraperitoneally in 2-ml volumes. For the assay of each solution three rats were used; all solutions were assayed at the same time.

The results obtained demonstrate that at the 5.0-mg level the 16-C^{14}-labelled conjugate was not estrogenic. It should be noted, however, that the dosage of conjugate assayed possessed a steroid content equivalent to 450 μg of

estrone, i.e., over 2000 times the amount of free estrone employed as standard. Even on the assumption that only one steroid residue was active per molecule of conjugate, this would still give some 100 times the equivalent of estrone required for appreciable uterine response. The fact that no increase in U/B ratio was observed for the conjugate at the 5-mg level is, therefore, strongly indicative of an absolute lack of estrogenicity. In contrast, the C17-amino and C17-glycine – ureido derivatives of estrone were active at the 40 μg and possibly at a lower level (no attempt was made to determine the potency of these compounds in relation to estrone).

The estrogenicity of estrone-17-carbamido-glycine is particularly noteworthy since this derivative contains the same linkage as that of the estrone-protein conjugates. This indicates that the lack of estrogenicity of the conjugates at the dosage level employed (5 mg) was not due to the carbamido function at the C17-position of the estronyl residue but to conjugation of the steroid to protein.

On the basis of these results, it is tempting to suggest that the hormonal activity of steroids is a property of the free, unconjugated steroid molecules or of steroid derivatives of sufficiently low molecular weight to permit diffusion of these derivatives into the cells of the target organs. The lack of hormonal activity of the estrone-17-carbamido-protein conjugate may, therefore, be attributed to the stability of this conjugate in vivo, i.e. to the resistance of the carbamido linkage to enzymatic breakdown. In this connection it should be noted that Lieberman *et al.* (8) reported that their estrone-17-O-carboxymethyl-oxime-BSA conjugate possessed estrogenic activity at 1.5-mg dosage level administered to immature rats over a period of 6 days. As was also suggested by these authors, the hormonal activity of their conjugates may have been due to enzymatic splitting of the estronyl residues from the carrier protein.

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ANTIBODIES TO ESTRONE-PROTEIN CONJUGATES

III. TISSUE LOCALIZATION OF ESTROGENS[1]

L. GOODFRIEND,[2] A. LEZNOFF,[3] AND A. H. SEHON

Abstract

Evidence was obtained that the mature rat ovary contained cellular component(s) which cross-reacted with fluorescein-labelled antiserum to estrone-2-carbamido-HSA.

The preparation of antiserum specific to steroid residues of synthetic estrone-protein conjugates (1, 2) opened up the possibility of localizing estrogenic steroids in tissues by the fluorescein-labelling technique (3). Localization depends upon the formation of antigen-fluorescent antibody aggregates in sufficient amounts to make fluorescence discernible. This makes the detection of small, univalent molecules such as estrone or estradiol problematical since the latter do not form precipitates with antibody. The possibility cannot be overlooked, however, that in some tissues the steroid might be firmly fixed to cellular components in sufficiently high concentration for localization. The localization of estrogens in the rat ovary was, therefore, attempted with fluorescein-labelled antibodies to estrone-2-carbamido-HSA.*

Materials and Methods

1. Preparation of Estrone-2-carbamido-HSA (C2-E-HSA)

The immunizing antigen, C2-E-HSA, was prepared according to the reaction sequence shown in Fig. 1. The 2-nitro derivative of estrone was prepared according to Werbin and Holoway (4) and the amine according to Kraychy and Gallagher (5). The conditions of phosgenation for the formation of estrone-2-isocyanate were identical with those described for the preparation of estrone-17-isocyanate (6).

An attempt was made to characterize estrone-2-isocyanate by forming the corresponding glycine ureide; however, the latter was not isolated in sufficiently pure form for satisfactory elementary analysis. The isocyanate was reacted with HSA to form esterone-2-carbamido-HSA (C2-E-HSA) and the conjugate was purified by methods described for the purification of C17-estrone-protein

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*NOTE: HSA = human serum albumin.

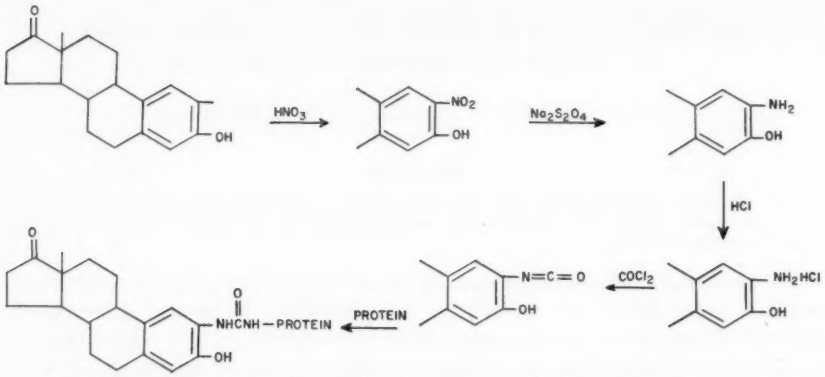

FIG. 1. Flowsheet for the preparation of estrone-2-carbamido-protein conjugates.

conjugates (1, 6). By free amino and ultraviolet spectrophotometric analysis (see (6) for methods), 35 estronyl residues were found coupled per molecule of HSA. Moreover, in 0.1 *M* phosphate buffer at pH 6.0 the electrophoretic mobility of the conjugate was -8.12×10^{-5} cm²/volt sec, compared to -4.31×10^{-5} cm²/volt sec for the control HSA (1).

2. Antiserum to C2-E-HSA

Rabbits were immunized with C2-E-HSA according to the procedure employed with the C17-conjugate (1). The sera after "booster" immunization were pooled. Evidence for the production of two types of antibodies, i.e. antibodies directed to the carrier protein, HSA, and to the estronyl residues of the immunizing conjugate, was obtained by means of the quantitative precipitin technique (1) and the results are shown in Table I. Following absorption of the antiserum with HSA, the antiserum still reacted with the conjugate (see Table I) but not with control HSA or α-naphthyl-HSA prepared as described previously (1). Further demonstration of the estrone specificity of the antibodies present in the antiserum absorbed with HSA was obtained by hapten-inhibition experiments using 2-amino-estrone and 2-nitro-estrone. As can be seen from the results listed in Table II, the steroids caused a marked reduction in the amount of precipitate compared to the uninhibited reaction. On the other hand, no inhibition was found on addition of these steroids to a control system consisting of HSA and the unabsorbed antiserum to C2-E-HSA.

3. Fluorescein Labelling

Antiserum to C2-E-HSA and antiserum to human gamma globulin (HGG), the latter serving as a control, were used in these experiments. The antibody content of the antiserum to C2-E-HSA was about 2.5 mg/ml.* The antibody content of the antiserum to HGG was not determined, but the serum was found to react strongly by ring test with the corresponding antigen.

*This value was estimated from the maximum antibody nitrogen precipitated by C2-E-HSA conjugate from antiserum to C2-E-HSA which was previously absorbed with HSA (see Table II).

TABLE I

Reactions of control HSA and C2-E-HSA with antiserum to C2-E-HSA by quantitative precipitation

Antigen	μg antigen N added	μg N precipitated with:	
		Antiserum*	Antiserum absorbed with HSA*
HSA	0.875	9.8	0
	1.75	16.3	0
	3.5	27.5	0
	7	28.5	0
	14	15.3	0
	28	11.5	0
	56	7.3	0
C2-E-HSA	1.5	22	13
	3	35	31.5
	6	56	62.0
	12	93	86.0
	24	114	95.0
	48	110	65.0
	96	64	31.5

*The antiserum was diluted fourfold with borate buffer (0.1 *M*, pH 8.0).

TABLE II

Hapten inhibition of precipitation of an anti-C2-E-HSA serum absorbed with HSA

C2-E-HSA added (μg N)	μg N precipitated		
	Uninhibited	Inhibited with 0.35 μmole of 2-amino-esterone	Inhibited with 1.24 μmoles of 2-nitro-estrone
5.125	39.0	13.0	6.0
10.25	68.0	19.5	10.0
20.5	85.0	17.0	10.5
41	73.5	11.0	8.0

NOTE: The antiserum was diluted fourfold with borate buffer (0.1 *M*, pH 8.0). For the uninhibited and inhibited reactions, 2.0-ml volumes of buffer and steroid in buffer were added per tube, respectively.

Crude globulin fractions of the sera were prepared by 50% ammonium sulphate precipitation and were subsequently conjugated with fluorescein isothiocyanate* according to the method of Riggs (7). To remove uncoupled fluorescein isothiocyanate, the conjugated globulins were absorbed twice with activated charcoal (15 mg per ml of fluorescent globulin solution). The procedures for absorption were identical with those described by Coons (3) except for the use of charcoal in place of mouse liver powder. The absorbed fluorescent globulin preparations were frozen in small aliquots and stored at −20° C until used.

4. Histological Procedure

Immediately after extirpation, rat ovaries were frozen in liquid nitrogen and stored at −20° C. Sections were prepared and stained according to the method of Vasquez and Dixon (8). All slides were mounted in buffered glycerine and viewed with a Leitz fluorescence microscope.

*Prepared from fluorescein amine by the method of Riggs (7).

Experimental Results

Tissue slices from the ovaries of two mature female rats and of two immature female rats, used as controls, were stained with tagged anti-C2-E-HSA globulin. Parallel tissue slices were stained with tagged anti-HGG globulin as controls. The control tissues stained with either globulin preparations showed no fluorescence. The ovarian tissues from one mature rat showed green fluorescence in the cytoplasmic region of perifollicular cells (Fig. 2), when stained with tagged anti-C2-E-HSA globulin, while no fluorescence was observed with tissues from the same ovary stained with tagged-HGG globulin. The ovarian tissues from a second mature rat showed no fluorescence on staining with the estrone-specific globulin. On the supposition that this negative result might be connected with the time of the oestrus cycle at which the animal was sacrificed, it was decided to prime mature female rats with pig follicle-stimulating hormone (FSH), obtained from Nordic Biochemicals, Montreal. Three rats were injected intramuscularly on 8 successive days with a total of 12 mg of FSH and sacrificed. The uteri were found to be highly swollen and the ovaries showed greatly enlarged follicles. Ovarian tissue slices from all three rats gave the same results on staining with tagged anti-C2-E-HSA globulin, namely, fluorescence of perifollicular cells as with the mature ovary noted above. In addition, bright fluorescence of stroma cells throughout the ovary (Fig. 3) was observed, indicating more extensive binding of the fluorescein-labelled antibodies to the ovarian tissue.

Discussion

The results obtained from fluorescence localization experiments with mature rat ovary demonstrated the presence of some tissue component reacting with antibodies to C2-E-HSA in perifollicular cells, and in the perifollicular and stroma cells of FSH-stimulated ovaries. It is clear, however, that further experiments would have to be made to determine if this component is identical with an estrogen–tissue complex. Such experiments would include, for example, (i) staining with tagged heterologous antisera other than that employed, including antiserum to HSA; (ii) specific inhibition of fluorescence by tagged antiserum to C2-E-HSA previously incubated with free estrone; (iii) staining of mature ovaries through the entire oestrus cycle.

In spite of the incompleteness of this study it is of interest to note that the cells which appeared to take up the fluorescein-labelled antibodies to estrone-protein conjugates are also believed to be involved in the production of estrogens (9).

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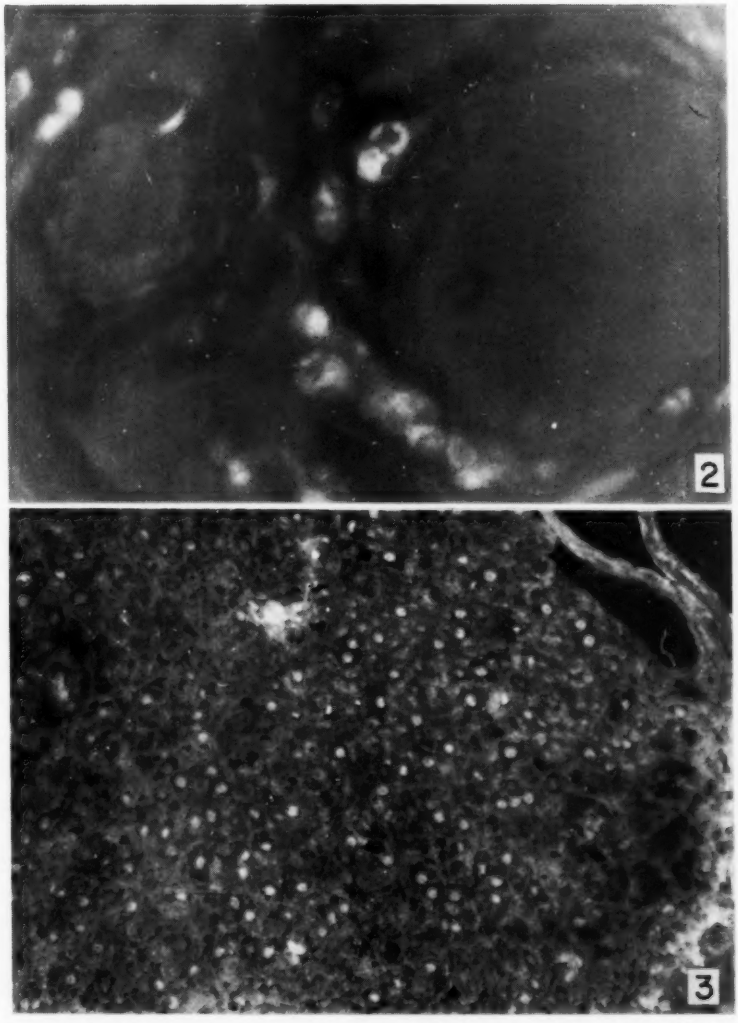

FIG. 2. Section of mature rat ovary stained with fluorescein-labelled anti-C2-E-HSA globulin. Perifollicular cells showed green fluorescence of the cytoplasm.

FIG. 3. Section of ovary from FSH-stimulated mature rat stained with fluorescein-labelled anti-C2-E-HSA globulin.

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NOTES

IDENTIFICATION OF A STEROID GLYCOL PRODUCED BY HUMAN OVARIAN SLICES FROM 17α-HYDROXYPROGESTERONE*

THOMAS SANDOR AND ANDRÉ LANTHIER

We have previously reported the isolation and partial chemical characterization of two steroid glycols originating from in vitro systems using Δ^4-pregnene-17α-ol-3,20-dione (17α-hydroxyprogesterone) as substrate and surviving human ovarian slices as the source of enzymes. The two glycols, designated as X_1 and X_2, were assigned the following formulae: X_2 was found to be identical with synthetic Δ^4-pregnene-17α,20β-diol-3-one, while on the basis of indirect evidence it was suggested that X_1 might be the 20α epimer of X_2 (1, 2).

Recently, through the courtesy of Dr. A. Nussbaum of Schering Corporation, Bloomfield, N.J., some synthetic Δ^4-pregnene-17α,20α-diol-3-one was obtained and the chemical identity of X_1 was reinvestigated.

A comparison of the chemical and physicochemical characteristics of X_1 and the authentic Δ^4-pregnene-17α,20α-diol-3-one gave the following results.

1. Chromatographic Mobilities

In mixed chromatograms in the paper chromatographic systems petroleum ether/80% methanol in water (3), isooctane–toluene (1:1)/70% methanol in water (3), cyclohexane–benzene (1:1)/ethylene glycol (2), X_1 and Δ^4-pregnene-17α,20α-diol-3-one did not separate from each other.†

2. Acetylation (2)

Both compounds formed acetates after treatment with acetic anhydride – pyridine (3:1) for 48 hours at room temperature. The resulting acetates had identical mobilities in the paper chromatographic system petroleum ether/70% methanol (mobility of acetate relative to 17α-hydroxyprogesterone, 2.70).

3. Absorption Spectrum in 95% Ethanol

Both compounds showed a maximal absorption at 240 mμ.

4. Absorption Spectrum in Concentrated Sulphuric Acid (4) (see Fig. 1)

(A) Reaction time: 30 minutes at room temperature.

X_1: Maxima: 292, 330, 410 *I* mμ
Minima: 235, 325 mμ

Authentic Δ^4-pregnene-17α,20α-diol-3-one: Maxima: 290, 335, 425 mμ
Minima: 235, 320, 390 mμ

*This work was supported by a research grant (A-4330) from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

†Substances were detected on chromatograms with an ultraviolet light source (Mineralight, R.V. 41, Research Equipment Corp., California: maximal emission, 254 mμ).

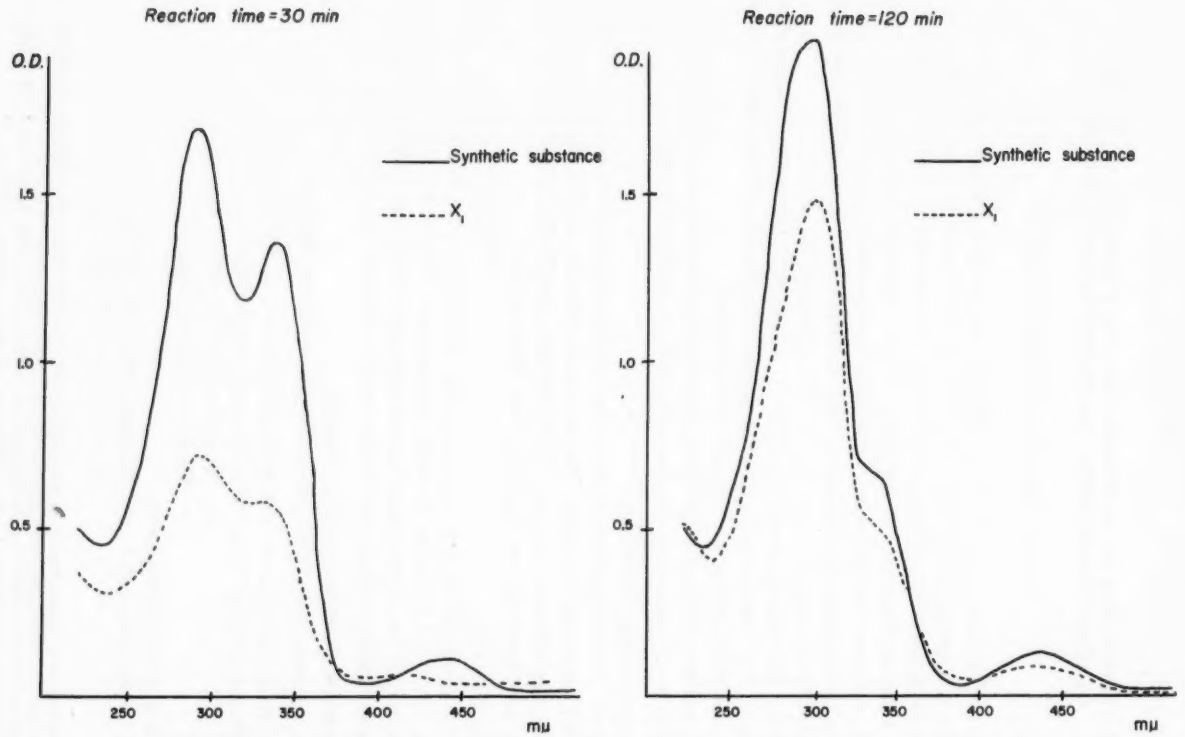

FIG. 1. This figure shows a comparison of the absorption spectra in concentrated sulphuric acid of biosynthetic X_1 and authentic Δ^4-pregnene-17α,20α-diol-3-one (synthetic substance). The spectra were determined in a Beckman DU quartz spectrophotometer using matched silica cells with a 10-mm light path.

(B) Reaction time: 120 minutes at room temperature.

X_1: Maxima: 300, 335 *I*, 435 mμ
Minima: 240, 390 mμ

Authentic Δ^4-pregnene-17α,20α-diol-3-one: Maxima: 300, 335 *I*, 440 mμ
Minima: 235, 390 mμ

5. Absorption Spectrum in "100%" Phosphoric Acid (5)

X_1: Maxima: 235, 280, 410, 485 *I* mμ
Minima: 225, 250, 350 mμ

Authentic Δ^4-pregnene-17α,20α-diol-3-one: Maxima: 235, 285, 410, 480 *I* mμ
Minima: 250, 350 mμ

6. Oxidative Degradation

Oxidation with both CrO_3 in glacial acetic acid (6) and periodic acid (6) produced Δ^4-androstene-3,17-dione from both compounds. The dione was identified on the strength of mixed chromatograms with authentic Δ^4-androstene-3,17-dione in three paper chromatographic systems; positive Zimmermann reaction (7) and absorption spectra in 95% ethanol and concentrated sulphuric acid (2).

The data presented above confirm the original suggestion that X_1 is identical with Δ^4-pregnene-17α,20α-diol-3-one.

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